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Vol. 41 November, 1958 Number 11

CONTENTS

People and Events 3

President K. L. Turk Comments 3

Vice-President A. C. Fay Writes 5

From the Desk of H. F. Judkins 21

Research Papers:

Some Electrophoretic Characteristics of Two Protein Components of the Fat-Globule Membrane of Normal Cow's Milk. J. R. Brunner and C. T. Herald 1489

A Chromatographic Technique for the Estimation of Some of the Free Fatty Acids in Lipolyzed Dairy Products. A. R. Kemp and J. H. Hetrick . 1494

A Gas-Liquid Chromatographic Method for Volatile Fatty Acids in Milk. C. L. Hankinson, W. J. Harper, and E. Mikolajcik 1502

A Resazurin Disk Assay Method for Detecting Antibiotics and Natural Starter Inhibitory Activity in Milk. K. M. Shahani and M. C. Badami . . . 1510

A Comparison of Bacterial Growth in Milk and in Milk-Water Combinations. T. J. Claydon, V. D. Foltz, and H. C. Fryer 1518

Bovine Fetal Hemoglobin. I. Postnatal Persistence and Relation to Adult Hemoglobins. R. M. Grimes, C. W. Duncan, and C. A. Lassiter . . 1527

Interrelationships of Milk Temperature, Dilution, and Curd Formation in the Response of Calves to Whole Milk Diets. F. G. Owen and C. J. Brown . 1534

Factors Associated with Estimated Fertilization and Service Efficiency of Cows. R. E. Erb and E. W. Holtz 1541

Effect of Method of Glycerol Addition on the Fertility of Frozen Bovine Spermatozoa. E. F. Graham, D. W. Vogt, and G. R. Fisher 1553

Efficacy of Oat Hay as a Legume Bloat Preventive in Cattle. H. W. Colvin, Jr., P. T. Cupps, and H. H. Cole 1557

Dietary Influences on Eructation and Related Ruminal Phenomena in Cattle. H. W. Colvin, Jr., P. T. Cupps, and H. H. Cole 1565

Some Observations on the Use of Sodium Propionate for the Prevention of Ketosis in Dairy Cattle. C. R. Richards 1580

Production of Gas in Rumen Fermentations and Stable Mass Formation from Alfalfa Fiber and Pectic Substances. H. R. Conrad, W. D. Pounden, O. G. Bentley, and A. W. Fetter 1586

Influence of Purified Materials on the Development of the Ruminant Stomach. W. P. Flatt, R. G. Warner, and J. K. Loosli 1593

Short-Interval Milking as a Physiological Technique. I. Effect of Frequent Milking with the Aid of Oxytocin on Milk and Milk Fat Production. S. Lakshmanan, J. C. Shaw, R. E. McDowell, M. F. Ellmore, and M. F. Fohrman 1601

Variation in the Thyroxine-Secretion Rate of Cattle. B. N. Premachandra, G. W. Pipes, and C. W. Turner 1609

(Continued on inside front cover)

CONTENTS

(Continued from front cover)

Effect of Molasses and Bacitracin on the Estrogenic Activity of Silage. F. N. Andrews and Martin Stob 1616

Effect of Natural Combinations of Ambient Temperature and Relative Humidity on the Water Intake of Lactating and Nonlactating Dairy Cows. Ralph Harbin, F. G. Harbaugh, K. L. Neeley, and N. C. Fine 1621

Repeatability Estimates of per Cent Total Protein and Certain Other Constituents of the Milk of Holstein Cows at Selected Intervals of Lactation. S. D. Musgrave, R. E. Walton, F. A. Graybill, R. Macvicar, J. B. Mickle, and W. E. Hardy 1628

Technical Notes:

Results of a Blood Test for Brucellosis and Leptospirosis in a Large Dairy Herd. R. R. Shrode, M. A. Brown, and H. E. Redmond 1633

A Comparison of Egg Yolk-Sodium Citrate, Egg Yolk–Sodium Citrate, Egg Yolk–Sodium Citrate–Glycine, and Egg Yolk–Glycine as Semen Diluters. S. K. Saha and R. B. Singh 1633

Our Industry Today:

Growth and Development of the Dairy Industry in the Union of South Africa. S. Bakalor 1636

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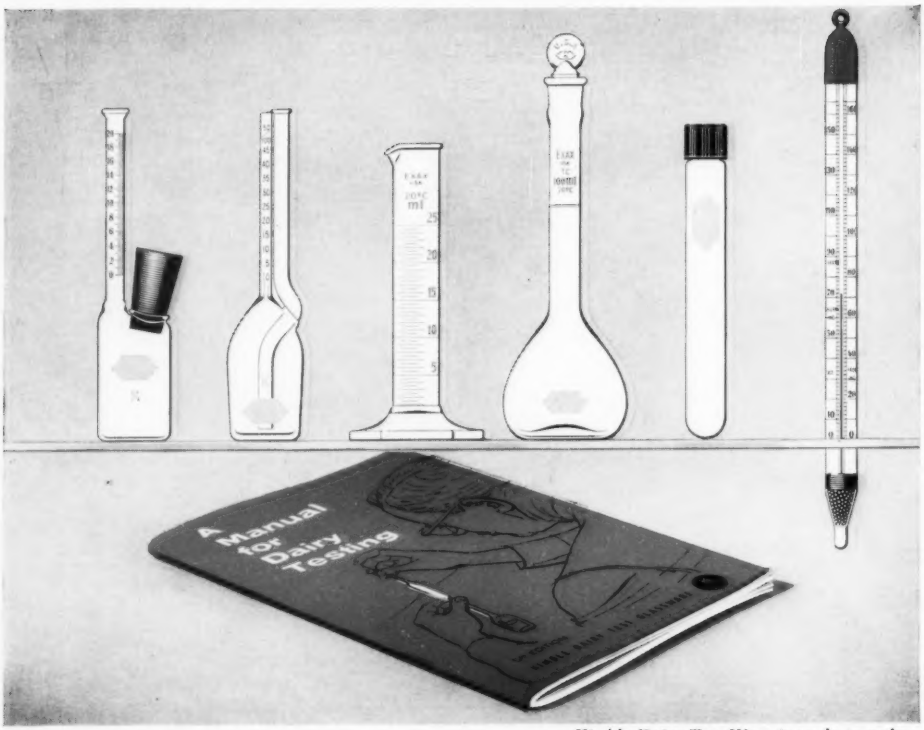

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PEOPLE AND EVENTS

Nominating Committee Solicits Vice-President, Director Nominees

In accordance with the policy approved by Board of Directors, the Nominating Committee solicits your suggestions for nominees to the offices of Vice-President and Directors. Please send your suggestions to me by January 1, 1959.

When your suggestions have been received, you will be sent forms on which to provide biographical data for your nominee.

Your help will enable your committee to submit a better slate of officers, so please let us have your suggestions.

W. M. ROBERTS, *Chairman*
A.D.S.A. Nominating Committee,
Dairy Manufacturing Section,
North Carolina State College,
Raleigh, North Carolina

Call for Papers for the 1959 Annual Meeting of the American Dairy Science Association

The 54th annual meeting of the American Dairy Science Association will be held June 15, 16, and 17, 1959, at the Univ. of Illinois, Urbana.

Members who wish to present papers must furnish titles and abstracts not later than Feb. 1. This deadline must be met so that the program can appear in the April Journal and the abstracts in the May Journal.

Abstracts must not contain over 200 words, arrived at by counting each typed line as 12 words. Abstracts longer than 200 words will be returned to the Author(s) by the Committee. An original and three copies of each abstract should be prepared and typed double-spaced. The original and one copy should be mailed to the Chairman, another copy to the Vice-Chairman, and the fourth copy to the Secretary of the Section before which the paper is to be presented.

In the preparation of abstracts, the Journal style and abbreviations must be used. Please refer to the October 1957 issue of the JOURNAL OF DAIRY SCIENCE for detailed suggestions for abstract or summary preparation. The title of the abstract should indicate clearly the nature of the research. Complete data should be presented showing the major results of the research. Essential but not excess statistics will make the data more understandable.

All members of the Association are encouraged to present papers. Participation by members in industry and senior members of the Association are particularly welcome. Abstracts of research already submitted for publication are not acceptable. The Program Committee favors the general policy that an individual be limited to the presentation of one paper or appear as an author on not more than two papers. The majority of the membership has accepted this policy recognizing that it limits the reporting of data by full-time research workers and by departments devoting much of their time to research. Your Program Committee and Association Members desire to stimulate sound research and improve the quality of papers presented. They believe that the reporting of scientific results on a friendly, yet competitive basis is an important part of the Annual Meeting. Your Program Committee will permit an individual to submit the number of papers he desires, provided that if he exceeds the number stated in the present policy his department must rate the abstracts in order of current importance, value, and contribution to dairy science. The Section Program Committee reserves the right to reduce the number of papers submitted by an individual.

It is preferable to present data in mimeographed form rather than by use of slides. Material for projection should be used only if the preferred method is impossible. About 250 mimeographed copies of data, graphs, pertinent information, etc., should be prepared. Authors can save much work and delay by making certain that their abstracts are submitted correct in every detail.

Names and addresses of officers of sections to whom titles and abstracts should be sent are:

Extension Section—L. A. JOHNSON, Dairy Dept., Michigan State Univ., East Lansing, chairman; C. W. NIBLER, Dairy Husbandry Dept., Univ. of Nebraska, Lincoln, vice chairman.

Manufacturing Section—B. W. WEBB, Research Laboratories, National Dairy Products Corporation, Oakdale, L.I., N.Y., chairman; STUART PATTON, Dairy Science Dept., Pennsylvania State Univ., University Park, vice chairman; and BURDET HEINEMAN, Producer's Creamery, Springfield, Mo., secretary.

Production Section—N. L. JACOBSON, Animal Industry Dept., Iowa State College, Ames, chairman; G. W. TRIMBERGER, Animal Industry Dept., Cornell Univ., Ithaca, N.Y., vice chairman; and S. D. MUSGRAVE, Dairy Husbandry Dept., Oklahoma State Univ., Stillwater, secretary.

The President Comments

Our membership campaign is now in full swing and we hope that each and every one of you has sent the names of several prospective members to our Secretary or to your state chairman of the Membership Committee.

Plans are moving ahead rapidly in the development of the program for the next annual meeting.

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Board of Directors approved the plan for our members to subscribe to *Dairy Science Abstracts*. This decision was made after slightly over 700 of our members replied to a questionnaire indicating they would subscribe.

This is a fine proposition for all of us to get the abstracts for a price of $4.90 a year against their regular price of $9.80. *Dairy Science Abstracts* is prepared and published by the Commonwealth Agricultural Bureaux in England. It gives a much greater coverage of scientific literature both in the United States and abroad than we now have in our abstract section of the Journal.

Under this plan for making *Dairy Science Abstracts* available to all of us, it obviously would be unwise to continue the present abstracts in our Journal. As a result, our present abstract section will be discontinued after the December, 1958, issue. I should like to point out, however, that you will not be short changed on material. Other material will be placed in our Journal to replace the space now being used for the abstracts.

Along with your dues notice for 1959 from our Secretary, you have received a notice concerning your subscription to *Dairy Science Abstracts*. Please send your check for $4.90, along with your check for your dues, to Mr. H. F. JUDKINS, 32 Ridgeway Circle, White Plains, N.Y. The publisher will mail the Abstracts monthly direct to all subscribers without cost to our Association.

Our Secretary needs to collect the subscription, pay for at least 750 copies, and send the publishers the names and addresses of subscribers prior to Jan. 1. If you haven't already done so, you can do a good turn by sending in your check now.

Amendments to the Constitution: Along with your membership dues notice, you received a ballot to vote on some minor changes in the wording of some of the By-laws of our Association. These changes must be approved by 25% of the membership so check to see if you have returned your ballot with payment of your 1959 dues.

The first of these changes deals with the "Award of Honor" which goes to one of our 25-year members who has made a distinguished contribution to the Association. The second is concerned with the granting of the title of Life Member for those who have retired from full-time employment in dairy science or the dairy industry. The last one moves up the time for suggestions for new officers to be turned over to the Nominating Committee from April 1 to Jan. 1. All of these changes, if approved by you and the other members, should be effective in the functioning of our Association.

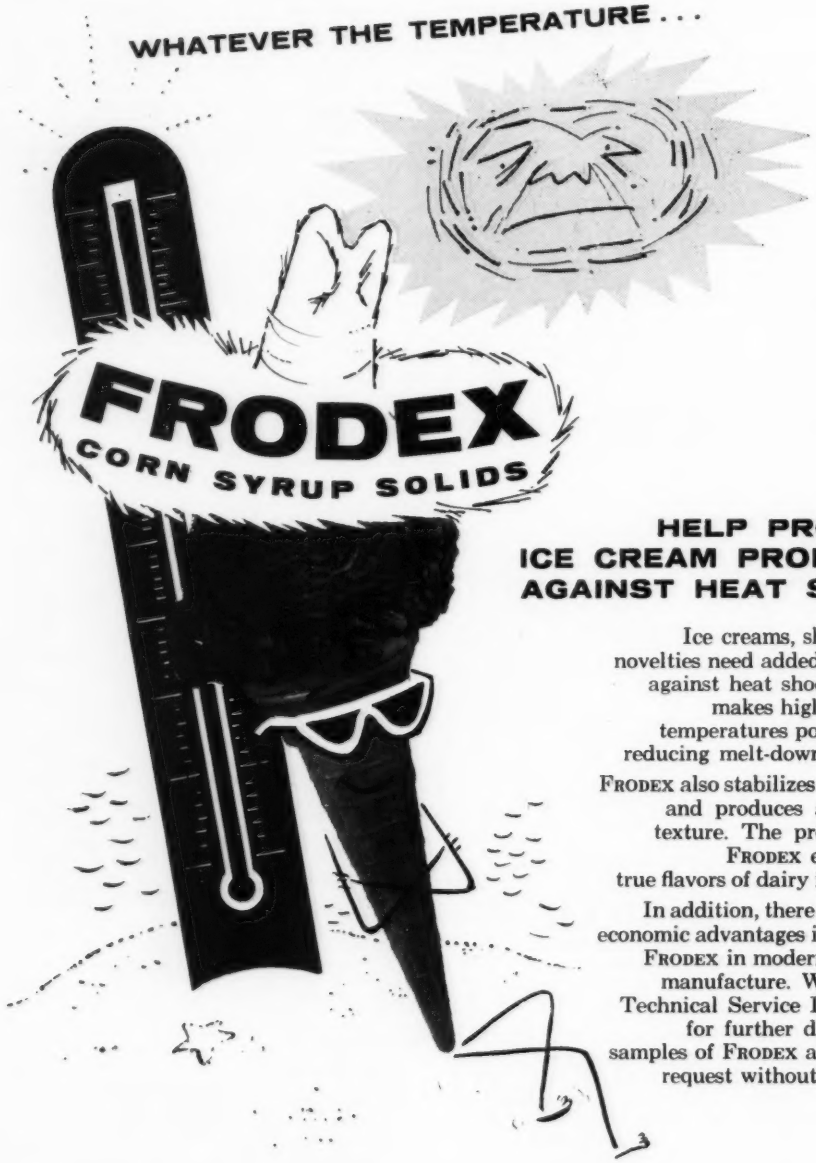
K. L. TURK,
President, A.D.S.A.

The American Dairy Science Association and the Dairy Industry

In the October issue of the Journal, President K. L. TURK has urged all members of the American Dairy Science Association to consider themselves members of the Membership Committee, and to be constantly on the alert for prospective new members. It has been said that we have reached the saturation point and that prospects are scarce. This is far from true.

To be sure, a substantial percentage of the eligibles connected with our state colleges and universities already belong to the American Dairy Science Association. There are a great many prospective members in industry. They are interested in the technical aspects of feeding, breeding and management of dairy herds, in the processing, marketing and inspection of dairy products, and in the manufacture and sale of equipment and supplies. This interest makes them likely candidates for membership. Not only would these people profit by membership in the American Dairy Science Association, but the Association would be strengthened by the contributions they could make in the practical consideration of technical problems.

The American Dairy Science Association is an organization dedicated to the task of promoting scientific growth and development of the dairy industry. It is made up largely of



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people who are devoting their lives to the study of fundamental and practical problems affecting this industry. Among its past and present members are some pure scientists who have contributed and are now contributing richly to fundamental concepts and new discoveries which are absolutely essential to progress. Also among its membership are many people whose thorough scientific training enables them to understand and interpret the basic principles discovered by the pure scientists and apply these principles to practical use by the industry. The American Dairy Science Association provides the fertile soil, not only for the germination of new ideas, but also for the practical cultivation of them to the fruiting stage.

Each month the JOURNAL OF DAIRY SCIENCE presents the results of original research and technical and practical papers outlining and discussing the results of research work recently completed or currently in progress in governmental research agencies, state experiment stations, and in industrial laboratories. Through this publication—and among others—these servants of the dairy industry not only exchange ideas and inspiration with other technical workers, but also share the results with industry people. In too many instances it has been assumed that the JOURNAL OF DAIRY SCIENCE is designed strictly for professional scientists, and that the papers are "over the heads" of the practical workers in industry. To be sure, many of the research papers are of a highly technical nature, but there are many others of a distinctly practical nature. The research work supported by tax money and private industry and reported in the Journal not only reflects the current trends in technological progress of the dairy industry, but also charts the course for future advancement.

Whether a dairy company is large or small, it is good business judgment to have one or more people in the organization keep abreast of scientific progress. At the last annual meeting of the Association in Raleigh, N.C., there were over 200 papers presented. A substantial percentage of these papers dealt with subjects of immediate and practical value to some phase of the dairy industry. Some of these papers dealt with feeding, breeding, processing, nutrition, and marketing of dairy products. Whether a person's special interests were in fluid milk, cheese, butter, ice cream, dried milk, concentrated milk, or some other phase of the industry, numerous papers were presented which covered the fundamental and practical aspects of the subject.

In addition to hearing the papers and subsequent discussions, there is always the opportunity for personal and private contact with the persons who conducted the research. Such contacts are of inestimable value for future use in obtaining desired information.

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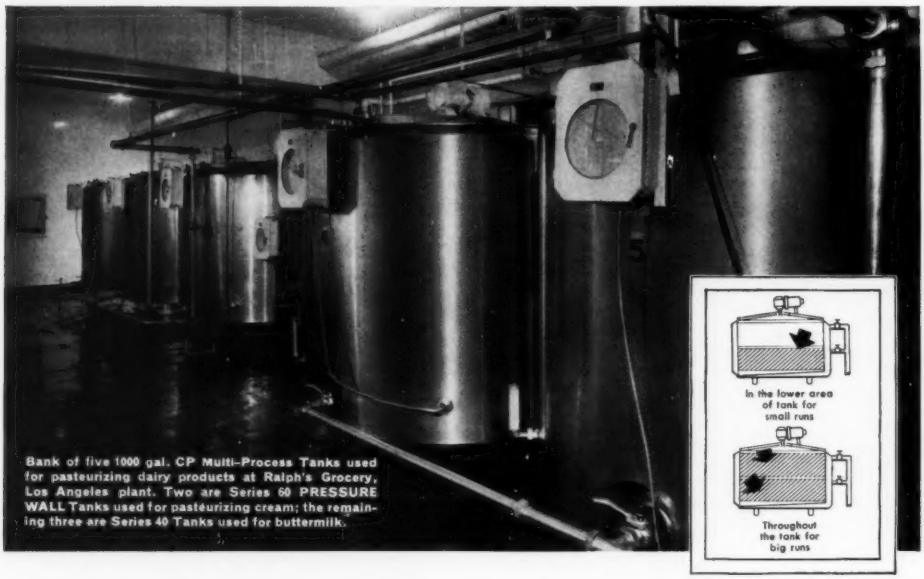

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dairy industry, whether they represent large or small companies. It is to the smaller and intermediate sized companies that this Association should have a particularly strong appeal. I firmly believe that the need for keeping abreast of technological developments and the value of help from outside contacts increase with decreasing size of a dairy company. Service is usually most valuable where it is most needed. I also sincerely hope that an increasing number of smaller and intermediate size dairy companies will not only take out membership in the American Dairy Science Association, but also will delegate one or more persons to attend the annual meetings. The larger dairy companies, too, may well consider the value of extending the advantages of membership to more of their people.

It is a sound business investment for any company, large or small, to encourage it's personnel to improve themselves and their service to the company. It is equally sound for the individual employee to make such an investment in himself.

These are some of the things to think about when it comes time to renew your own membership and to suggest membership to your associates in the broad field of dairy technology. People in industry need the services which the Association has to offer, and the Association needs a broader representation from industry. Look over the field of industrial prospects in your section of the country. It is far from true that the prospects are scarce.

A. C. Fay,
Vice-President, A.D.S.A.

Brown Tells About Dairying in Afghanistan

D. C. Brown, Univ. of Wyoming Dairy Dept., recently returned from a two-year tour of duty at Kandahar, Afghanistan, where he taught general agriculture at Ahmad Shah Baba College as a member of the University Team for Research and Education, United States Operations Missions to Afghanistan.

Although not assigned to a dairy program, Mr. Brown had many opportunities to visit dairies and observe production and marketing methods.

Cattle in Afghanistan are kept primarily for two purposes—milk or draft. Dairying and beef production as we know it in the United States is not practiced. Most of the country is better adapted to the grazing of sheep and goats. Hay, grain, and pasture production is limited; straw, moistened with water, often provides a good share of the ration.

In recent years there have been a few importations of better grade dairy stock for the government farms, but native cattle of Indian blood lines predominate. One of these breeds the Kandahari is better adapted to milk production and resembles the Red Sindhi. Very few

Hormones control blood and tissue levels of fats and related substances such as cholesterol . . . rate at which these are synthesized and destroyed . . . and manner in which they are utilized for body functioning. Fat metabolism is influenced by secretions of the pituitary, thyroid, adrenals, ovaries, testes and pancreas, and by action of liver and possibly kidneys.

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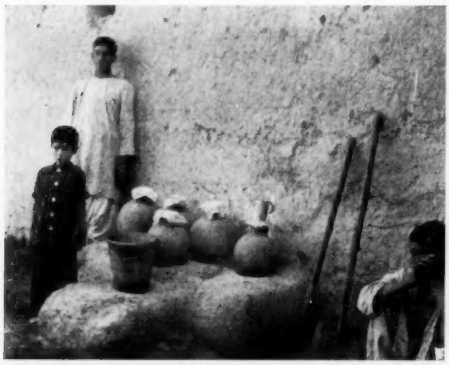
dairies of 25 or more cows are to be found. In the villages and near larger cities a few family cows are found. In the larger cities a few dairy food shops sell milk, buttermilk, butter oil (ghee), and curdled milk (mast). These products are bulk handled and consumers bring their own containers. Much of the ghee is produced from ewes milk and supplied by nomads who bring the product to market in goatskin containers.

Native Kandahari Cows feed on wet straw from the feed manger. During the day the cows are on pasture. These Afghan cows weigh about 700-750 lbs.

Dairy products are luxury foods and are relished by most Afghans who can afford them. Often, milk comes from sheep and goats rather than from cows. For many nomad tribes, dairy products furnish a good share of their diets.

The government is interested in promoting the dairy program, and at their experimental farm in Kabul, plans are under way for the installation of modern milking stalls and pasteurizing equipment as well as feeding and production experiments. A similar program is under way on the private farm of His Excellency, the King.



This photo shows Afghan milk jugs covered with cloth to keep out the flies. When milk is strained, cloth is used.

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1 **Finest ingredients.** Only top-grade cocoa and sucrose are used in Kraft's powder. We harvest our own Irish Moss, extract the stabilizer from it with our own careful process. Because all ingredients must meet our rigid specifications, you are assured uniformity of color, flavor and viscosity in the finished product.

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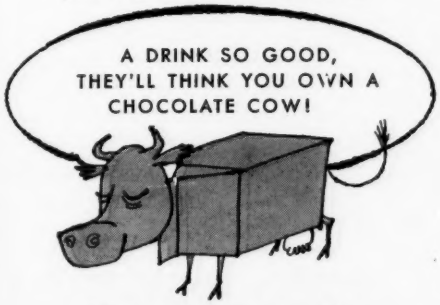
3 **Small batch manufacturing and testing.** No other manufacturer makes powder in such small batches. And a sample from every batch of Kraft's powder is actually made into chocolate milk and thoroughly tested before the batch is shipped.

Samples for classroom work made available to accredited dairy colleges. Just write your nearest Kraft division office.



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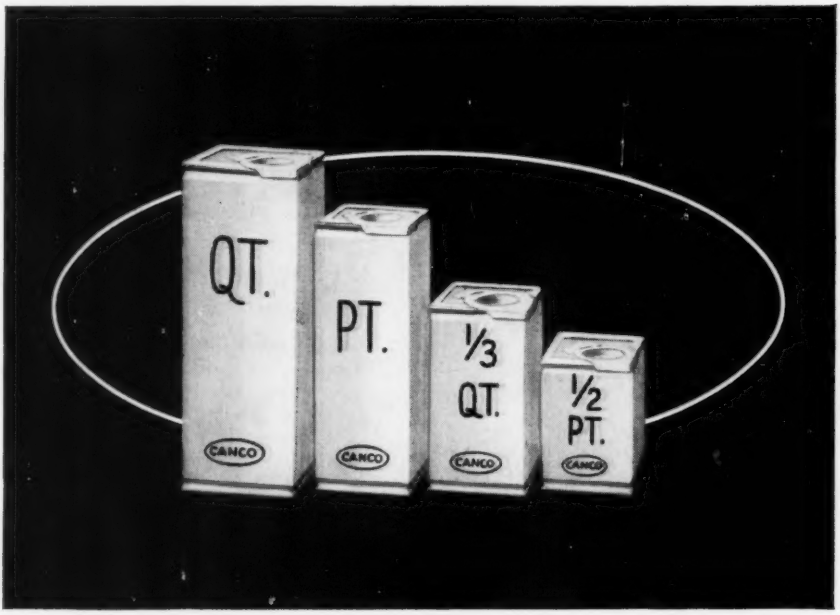
DIVISION OFFICES: NEW YORK; GARLAND, TEXAS; CHICAGO; SAN FRANCISCO



MILK

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Canco's paper containers, the original disposable cartons for milk, have been winning greater acceptance every year! Popular with homemakers everywhere, these containers are ideal for use in school cafeterias and vending machines. They are easy to open and close; provide "controlled pouring," and are compact, sturdy, sanitary. Yes, Canco cartons are the *preferred* containers for milk . . . the Grade "A" cartons for nature's most nearly perfect food!

CANCO AMERICAN CAN COMPANY

Much work needs to be done on a quality forage program. Alfalfa grows very well on most irrigated lands, and several cuttings a year are obtained. Since labor is cheap, green-feeding is practical. Efforts are being made to control the disease problem, namely, tuberculosis and Bangs disease. As soon as these efforts, plus a refrigeration and sanitation program, can be worked out, there is promise for an increase dairy program in the country.

An Afghan milks with the knuckle-forefinger milking technique. Note the calf tied at the cow's head. The calf nurses each quarter to stimulate milk let-down, then the milker takes over.

Minnesota News

ROBERT JENNESS, professor of agricultural biochemistry at the Univ. of Minnesota, has been granted special leave for the current term to do research at the Animal Proteins Laboratory of the Agricultural Research Service's Eastern Utilization Research and Development Division, Philadelphia.

Dr. Jenness, winner of the 1953 Borden Award in the Chemistry of Milk, has discovered a component of milk which he believes to be a hitherto unrecognized protein. The isolation and characterization of this milk protein will be the principal objective of his research for the ARS.

May, June '58 Journals Needed

Copies of the May and June, 1958, issues of the JOURNAL OF DAIRY SCIENCE are urgently needed to take care of back copy orders, late subscribers, and late memberships.

If you can spare these journals, send them to the JOURNAL OF DAIRY SCIENCE in care of The Garrard Press, 510 N. Hickory Street, Champaign, Illinois.

The Association will pay you $.50 for each copy you send plus actual shipping costs. As each shipment comes in, The Garrard Press will notify the Association of its contents, the sender, and the postage; the Association, in turn, will issue your check.

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4. Conduct research on new ideas suggested by you.

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SURGE

Kansas News

The Kansas Dairy Technology Society held a dinner meeting in November at Wichita. Speaker for the event was A. K. SAUNDERS, manager of the farm products division of the Diversey Corporation, Chicago. His topic was "Current Aspects and Problems of CIP Cleaning."

C. L. NORTON has been named head of the Dept. of Dairy Husbandry at Kansas State College, Manhattan. His appointment was effective Nov. 1. He will fill the vacancy created by the recent death of F. W. ATKESON.

Before going to Kansas State, Dr. Norton had been head of the Dairy Husbandry Dept. at Oklahoma State Univ., Stillwater. Prior to that he was head of animal and dairy husbandry at the Univ. of Rhode Island. Dr. Norton is a native of Illinois.

JERRY SLEEPER, plant superintendent at the Kansas State College Creamery, has resigned to take a position as Milk Sanitarian for the city of Atchison, Kan. Mr. Sleeper is a 1957 graduate of Kansas State in dairy manufacturing.

W. H. MARTIN, Dept. of Dairy Husbandry, Kansas State, participated in the program at the Univ. of Minnesota Dairy Products Institute held in September. His topics were "Cutting Cost, Not Quality of Ice Cream" and "How To Avoid Costly Shutdowns in the Ice Cream Plant."

Eastern A.D.S.A. Div. Meets in W. Springfield, Mass.

The 32nd Annual Meeting of the Eastern Division of the A.D.S.A. was held in September at West Springfield, Mass.

H. F. JUDKINS, Secretary-Treasurer of the A.D.S.A., reported on the national program of A.D.S.A. and the policies of the JOURNAL OF DAIRY SCIENCE.

J. E. KIRK, U.S. Dept. of Health, Education, and Welfare, reported on "Antibiotics in Dairy Products from the Public Health Standpoint." In his talk, he outlined the effective regulatory measures and public information programs being carried out by the Food and Drug Administration to prevent the occurrance of any public health problems from the presence of antibiotics in dairy products.

Papers were also presented by the New Jersey, Connecticut, New Hampshire, and Massachusetts Agricultural Experiment Stations.

Ali Baba *and the* 40 *Thieves*

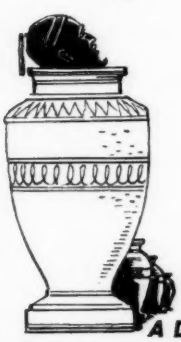
The fabled Ali Baba never suspected that he was harboring 40 armed thieves under his roof. The fable states that Ali's watchful servant, Morgiana, discovered them lurking there and saved Ali and his valuables.

There are 40 thieves lurking in every business operation today — awaiting every opportunity to rob it of profit. There are Wastes, Production Losses, Distribution Costs and a host of others — most of them well entrenched and not easily recognized.

That's why every business needs a watchful servant like Morgiana — a faithful guardian of profit. In the Dairy Industry that has been the function of the Dairy Manufacture and Research Bureau for 30 years

The Bureau uses a sharp and practised eye for ferreting out of Milk and Ice Cream operations the 40-odd leaks that may be robbing them of hard-won profit.

If you suspect **you** are paying tribute to some of these hidden thieves, why not join the Bureau **now**? Avail yourself of the Bureau's many services which will put your operation back on the road to **full** profit. For complete information, write



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The Intercollegiate Judging Contests sponsored by the Eastern State Exposition drew a total of 18 different college teams. In the judging, Cornell Univ. was the high team in both the dairy products and dairy cattle judging contests.

Results of the Intercollegiate judging were announced at the Annual Awards Banquet. Also featured at the banquet was the installation of the following officers: A. C. SMITH, Dept. of Animal Industries, Univ. of Connecticut, president; R. R. POULTON, Dept. of Animal Industry, Univ. of Maine, vice president, and F. E. POTTER, Dept. of Dairy and Animal Science, Univ. of Massachusetts, secretary.

Arizona Conference to Be Held Jan. 14-15

The Eighth Annual Dairy Industry Conference will be held at the Univ. of Arizona, Tucson, Jan. 14-15, 1959.

Guest speakers include P. H. TRACY, Univ. of Illinois; K. W. MICHAEL, Computer Dept., General Electric Co., Phoenix; JOSEPH CUNNINGHAM, Dairy Industries Supply Association, Washington, D.C.; J. L. ALM, Milk Plant Division, Safeway Stores, Inc., Los Angeles, Calif., and D. R. MOORE, Capital Business Service, Lansing, Mich.

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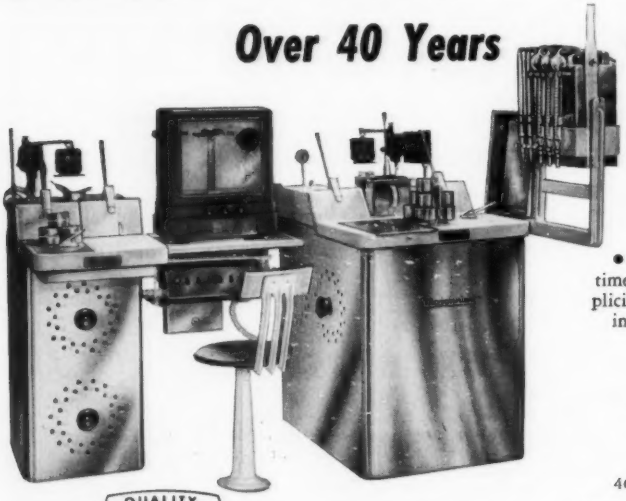

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B-B-L 01-245—**ENDO AGAR**
B-B-L 01-180—**EOSIN METHYLENE BLUE AGAR (LEVINE)**
B-B-L 01-269—**LAURYL SULFATE BROTH**
B-B-L 01-183—**TRYPTICASE GLUCOSE EXTRACT AGAR**

(1) Standard Methods for the Examination of Dairy Products, ed. 10, New York, American Public Health Association, 1953. (2) Committee Report, Am. J. Pub. Health 42:1131 (Sept.) 1952. (3) Microbiological Methods, report at 66th Ann. Meet. Assn. Official Agricultural Chemists, Sept. 29, 1952; J. Assn. Official Agr. Chem. 36:91 (Feb.) 1953. (4) Standard Methods for Examination of Water, Sewage and Industrial Wastes, ed. 10, New York, American Public Health Association, 1955.

Literature on B-B-L's **complete** line of culture media and apparatus for the microbiological laboratory sent on request. (13)

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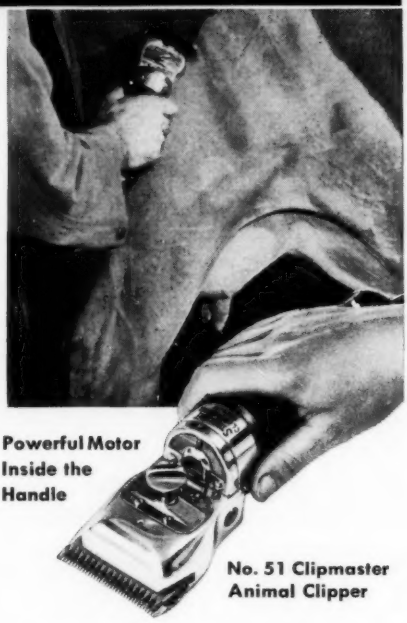

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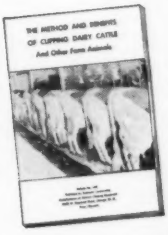

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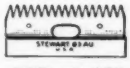

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Dairy Cattle Judging Results: National Intercollegiate Judging Contest

Thirty-one teams competed in the National Intercollegiate Dairy Cattle Judging Contest held at the National Dairy Cattle Congress, Waterloo, Ia.

Ten classes of four animals each, representing the five dairy breeds, were placed and oral reasons given on five of these classes. The contest was managed by R. C. FERGUSON, Meredith Publishing Co., Des Moines.

Official judges were JOE EVES, Meredith Publishing Co.; J. C. THOMPSON, Ralston-Purina Co., St. Louis, Mo.; EUGENE MEYERS, Hoards Dairymen, Ft. Atkinson, Wis.; R. D. STEWARD, American Guernsey Cattle Club, Peterborough, N.H., and A. C. THOMPSON, Elgin, Ill. The high team and high individual for each division are as follows:

All breed—Cornell Univ., high team; J. P. OTT, Mississippi State College, high individual.

Ayrshire—Cornell Univ., high team; JOHN MARBON, Cornell Univ., high individual.

Brown Swiss—Maryland Univ., high team; STAN SMITH, Kansas State College, high individual.

Guernsey—Ohio State Univ., high team; TED RIDGELY, Univ. of Maryland, high individual.

Holstein—Purdue Univ., high team; GARY JONES, Univ. of Minnesota, high individual.

Jersey—Mississippi State College, high team; JERRY ROBINSON, Louisiana State Univ., high individual.

Dairy Cattle Judging: Kansas State Fair Judging Contest

The Iowa State College Dairy Cattle Judging team won first place in four of six breed divisions and second in the other two divisions at the Sixth Intercollegiate Dairy Cattle Judging Contest held at the Kansas State Fair, Hutchinson.

Members of the Iowa team were D. EMMERT, J. SYNDERGAARD, H. TYRRELL, and C. ROGERS (alternate). Their coach is C. F. FOREMAN.

The Iowa team won not only the Kansas State Board of Agriculture Trophy for high team but also the F. W. ATKESON Memorial Award for the high team in oral reasons. The latter award was presented by Mrs. F. W. ATKESON and her son GEORGE in memory of the late F. W. ATKESON, of the two founders of this contest.

The Kansas Inter-Breed Dairy Cattle Council Cup was won by DAVID EMMERT of Iowa State, high man in the contest. Contestants from Iowa State, Kansas State College, Oklahoma State Univ., Colorado A & M, Univ. of Nebraska, Univ. of Missouri, and South Dakota State College were banquet guests of the Kansas Cooperative Dairy Products Association and the Kansas State Dairy Association.

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Dairy Cattle Judging Results: Southern Intercollegiate Contest

Eleven schools participated in the Southern Intercollegiate Dairy Cattle Judging Contest held at Memphis, Tenn., during September. Team placings in the contest are as follows:

Louisiana State Univ., Univ. of Tennessee, Mississippi State College, Univ. of Kentucky, Univ. of North Carolina, Univ. of Tennessee (Martin Campus), Western Kentucky State College, Texas A & M, Texas Technological College, and Clemson College.

The L.S.U. team placed first in judging Guernseys and first in judging Brown Swiss. The nearest competitor, Univ. of Tennessee, was 60 points behind the Louisiana team. GERALD PHARES, L.S.U., placed first in the individual judging of Brown Swiss.

Completed Theses

Ph.D. Degree

C. B. BROWNING—Effects of diethylstilbestrol on milk production in cows and rats, on certain changes in rumen ingesta, and on bovine differential leucocyte counts. Kansas State College, Manhattan.

E. E. ORMISTON—Some effects of inbreeding with selection on milk yield and growth in milk goats. Univ. of Illinois, Urbana.

MICAH KRICHEVSKY—Ribose-five-phosphate degradation in *microbacterium lacticum*. Univ. of Illinois, Urbana.

H. D. GRAHAM—Studies on the absorption of surface active agents by *staphylococcus aureus*. Univ. of Illinois, Urbana.

M.S. Degree

R. G. KINGWELL—Factors affecting the composition of bovine rumen gas. Univ. of Illinois, Urbana.

BERT LARK—The effect of stage of lactation on composition of milk of the dairy cow. Univ. of Illinois, Urbana.

P. M. T. HANSEN—A quantitative test for carrageenin ester sulphate in milk. Univ. of Illinois, Urbana.

Fifer Resigns as A.B.I. Chief

RUSSELL FIFER has resigned as executive secretary of the American Butter Institute, Chicago. He explained that "the dwindling butter production of major member companies made necessary certain economies which greatly limited and restricted my future with the organization."

After graduation from Ohio State Univ. in 1937, he performed supervisory work for the Borden Company and served as an assistant instructor and creamery manager in the University Dept. He also managed the Indiana and Ohio Dairy Products Associations.

His resignation from the A.B.I. comes after 13 years of service to the national butter industry.

FROM THE SECRETARY'S DESK

The following letter was sent to State Chairman on the Membership Committee regarding Necrology reports:

Heretofore the Association has had a small Necrology Committee designed to report deaths of members to the Secretary-Treasurer and to the Editor. This has involved a little 'red tape' and results have not been too satisfactory.

It occurred to me that since we had one man in each state as State Chairman for the membership campaign that we might ask these same men to report deaths of members that might occur in their respective states. Certainly you will be more likely to know of any such deaths than any small sectional committee.

President K. L. TURK has concurred in this plan. I'm sure you will be glad to cooperate in this important matter. I enclose herewith a few copies of the form on which deaths should be recorded in duplicate. Send both copies to me. I will retain one for my records and send the other to Editor E. O. Herreid for publication in the Journal. Should any deaths occur, please report them just as promptly as possible.

Each member should also consider himself as the one who can be helpful in reporting any deaths he may hear about in his state. Just send the following information in duplicate to the Secretary-Treasurer:

Name of deceased
Date of death
Birth date and place
Address of deceased
Name of nearest living relative
Address of nearest living relative
Education and training of deceased (schools attended giving dates and degrees received)
Accomplishments of deceased
Positions held
Relatives
Other remarks
Name of person reporting
Address of person reporting

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American Food Laboratories 7
American Maize-Products Company 6
Babson Bros. Company 14
Baltimore Biological Laboratories 17
Blumenthal Bros. Company 13
Creamery Package Company 8
The Dairy Laboratories 16
Difco ..... COVER 4
G. P. Gundlach & Company 15
Chr. Hansen's Laboratory 20
Johnson & Johnson 4
Kelco Company 5
Klenzade Products, Inc. 9
Kraft Foods 11
Lazarus Laboratories, Inc. 19
Marschall Dairy Laboratory, Inc. 19
David Michael & Company 20
Mojonnier Bros. Company 16
National Dairy Council 10
The Nestlé Company ..... COVER 3
Owens-Illinois 2
Sunbeam 18
Wisconsin Alumni Research Foundation 21

National Science Foundation Graduate and Postdoctoral Fellowships for 1959-60

The National Academy of Sciences–National Research Council will again assist the National Science Foundation with its eighth regular predoctoral and postdoctoral fellowship programs which have just been announced by the Foundation. The NSF plans to award approximately 1,000 graduate and 200 postdoctoral fellowships in these two programs for scientific study during the 1959-1960 academic year.

The evaluation of each candidate's application is made by the Academy–Research Council selection panels and boards. The National Science Foundation will make the final selection of Fellows and will announce the awards on March 15, 1959.

These fellowships are open only to citizens of the United States and are awarded solely on the basis of ability. The National Science Foundation has announced that ". . . fellowships will be awarded in the mathematical, physical, medical, biological, and engineering sciences, including anthropology and psychology (excluding clinical psychology), and from the following social sciences, where they conform to accepted standards of scientific inquiry by fulfilling the requirements of the basic scientific method as to objectivity, verifiability, and generality: geography, mathematical economics, econometrics, demography, information and communication theory, experimental and quantitative sociology, and the history and philosophy of science. Also included are interdisciplinary fields which are comprised of overlapping fields among two or more sciences."

Graduate fellowships are available to those who are working toward the master's or doctoral degrees in the first, intermediate, or terminal year of graduate study. College seniors who expect to receive a baccalaureate degree during the 1958-1959 academic year also are eligible to apply. Postdoctoral fellowships are available to individuals who, as of the beginning of their fellowship tenure, have a Ph.D. in one of the fields listed above, or who have had research training and experience equivalent to that represented by such a degree. In addition, holders of the M.D., D.D.S., or D.V.M. degree, who wish to obtain further training for a career in research, are eligible provided they can present an acceptable plan of study and research. "Awards are not made to individuals to pursue a course of study designed to prepare them further for careers in medical practice and comparable fields; however, applications will be accepted from those who intend to obtain further training in one of the medical sciences directed toward a career in research."

All applicants for graduate (predoctoral) awards will be required to take an examination designed to test scientific aptitude and achievement. This examination, administered by the Educational Testing Service, will be given on January 19, 1959, at designated centers throughout the United States and certain foreign countries.

The annual stipends for graduate Fellows are as follows: $1,800 for the first year; $2,000 for the intermediate year, and $2,200 for the terminal year. The annual stipend for postdoctoral Fellows is $4,500. Dependency allowances will be made to married Fellows. Tuition, laboratory fees, and limited travel allowances also will be provided.

Further information and application materials may be obtained from the Fellowship Office, National Academy of Sciences–National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D.C. The deadline for the receipt of applications for regular postdoctoral fellowships is December 22, 1958, and for graduate fellowships, January 5, 1959.

SOME ELECTROPHORETIC CHARACTERISTICS OF TWO PROTEIN COMPONENTS OF THE FAT-GLOBULE MEMBRANE OF NORMAL COW'S MILK[1]

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SUMMARY

Soluble proteins of the fat-globule membrane were studied electrophoretically in various buffer systems ranging from pH 2.05 to pH 8.79 at 1° C. One small and two large migrating boundaries were observed under conditions of maximum electrophoretic resolution.

The protein of the insoluble fraction was solubilized by protein-solubilizing agents such as sodium sulfide, sodium lauryl sulfate, or dodecyl benzene sodium sulfonate in alkaline solutions. Electrophoretic mobility patterns for these solubilized protein samples exhibited a single, homogeneous boundary for the sodium sulfide–treated protein, whereas the detergent-treated sample(s) were heterogeneous, showing one relatively small and two major migrating boundaries. The small boundary could be eliminated from the electrophoretic pattern by heating the solubilized protein solution to 75° C. momentarily.

Previous reports from this laboratory have indicated that the fat-globule membrane proteins(s) is composed of two fractions basically differentiated by selective solubility in 0.02 *M* sodium chloride solution *(4)*. The "soluble" fraction is soluble in all ordinary aqueous solvents; whereas, the "insoluble" fraction is insoluble in the same solvents, requiring the addition of alkaline sulfides or selected anionic detergents in alkaline solutions to effect solubilization.

Electrophoretic data characteristic of the soluble and solubilized insoluble protein fractions of the fat-membrane protein(s) of normal cow's milk are presented herein. These observations emphasize further the complex nature of the membrane proteins and for the first time characterize electrophoretically the recently recognized insoluble membrane protein fraction. Granted, the data presented here do not represent the ultimate characterization of the membrane components, but they do mark a significant step in this direction.

EXPERIMENTAL PROCEDURE

The fat-globule membrane material was isolated from the washed cream of unpasteurized, pooled milk, delipidified and separated into soluble and insoluble protein fractions according to the procedures already outlined *(4)*. In preparation for electrophoretic analysis, the soluble-fraction concentrate was diluted to approximately 0.5% protein with additional 0.02 *M* sodium chloride. Ten milli-

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[2] This article is a part of a dissertation presented to the Faculty of the School for Advanced Graduate Studies of Michigan State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] Present address: General Foods Corporation, Hoboken, New Jersey.

liters of the protein solution was dialyzed to equilibrium against three 400-ml. changes of selected buffer solutions at 5° C. The insoluble protein in 10 ml. of a 0.5% protein suspension in 0.02 *M* sodium chloride was solubilized by the following agents: sodium sulfide in a Na_2S/P ratio of 0.6:1, sodium lauryl sulfate in a D/P ratio of 0.8:1, or with dodecyl benzene sodium sulfonate in a D/P ratio of 1.2:1. These solubilized protein solutions were dialyzed at 5° C. to equilibrium against three 400-ml. changes of selected buffers. The solubilizing agents employed were selected from a group of alkaline sulfides, anionic and nonionic detergents as the most desirable agents to use under the limiting physical conditions encountered during electrophoresis. Some detergents capable of solubilizing the insoluble fraction into clear solutions at room temperature became cloudy or excessively viscous when the temperature of the solution was lowered to 1° C.

Electrophoretic characteristics of the soluble protein fraction were observed in the following buffer systems of 0.1 ionic strength: glycine—HCl, pH 2.05, and 3.78; acetate, pH 4.30 and 5.30; phosphate, pH 6.10 and 7.30, and veronal, pH 8.70. All pH values represent extrapolations to approximately 1° C. The solubilized insoluble protein fraction was examined in buffer systems alkaline to the isoelectric point (pH 5.0). Veronal buffer at pH 8.79 at 0.1° C. was most desirable for this study. In phosphate buffer systems similar patterns were obtained for membrane protein(s) samples isolated with *n*-butanol, although the migrating boundaries were more diffuse and migrated at a slower rate. These observations

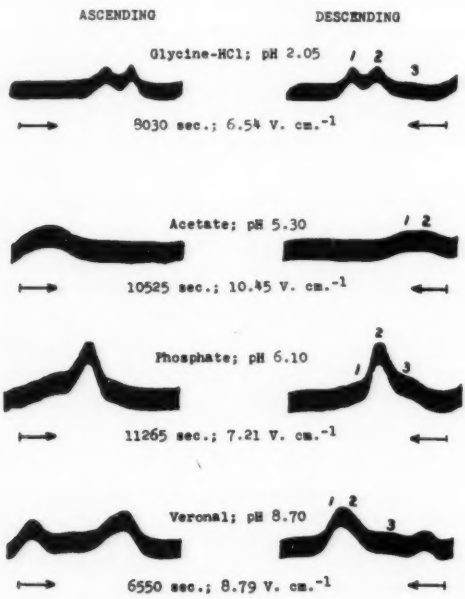

FIG. 1. Electrophoretic mobility patterns of the soluble membrane protein fraction in various buffers.

suggest that *n*-butanol and concentrations of ethanol greater than 35% have a denaturing effect upon the membrane-protein(s) when employed as lipoprotein cleavage agents. Also, the use of these agents at temperatures much above 0° C. resulted in a reduction in the electrophoretic mobilities of the proteins of the soluble fraction *(1)*.

A suggestion has been made that, in view of the procedure used in preparing these protein fractions (cold alcohol--ether treatment), there may be an artefact of casein involved. Isoelectric casein was prepared and treated in the same procedure as used for the preparation of the membrane fractions. Insofar as could be determined electrophoretically, casein treated in this manner and untreated casein showed identical patterns, adding to the evidence in support of the presence on the fat globule membrane of at least two protein components, differing widely in characteristics from one another and from casein.

The electrophoretic mobility patterns for the solubilized protein fraction in phosphate and veronal buffers are shown (Figures 2 and 3). Solubilization of the insoluble fraction with sodium sulfide resulted in the appearance of a homogenous migrating boundary; whereas, treatment with sodium lauryl sulfate

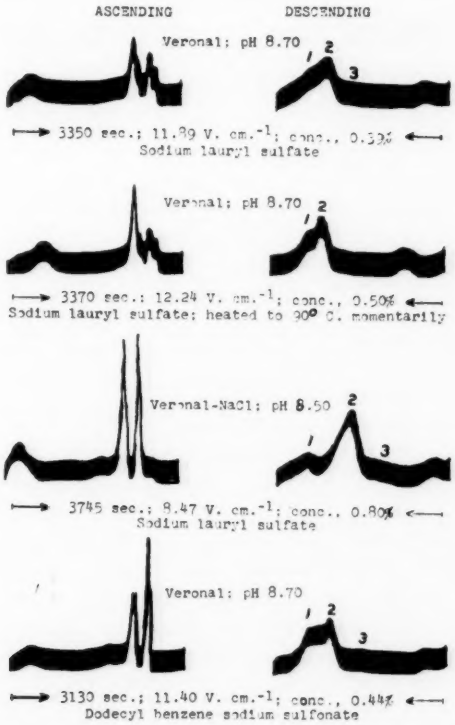

FIG. 2. Electrophoretic mobility patterns of the sodium sulfide—solubilized insoluble membrane protein fraction in phosphate and veronal buffers.

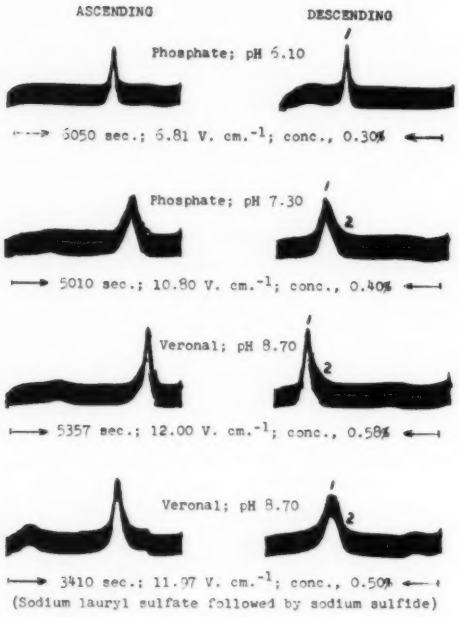

FIG. 3. Electrophoretic mobility patterns of the detergent-solubilized insoluble membrane protein fraction in veronal buffers.

or dodecyl benzene sodium sulfonate produced electrophoretic patterns containing two major heterogenous boundary areas and a slow-moving boundary of low concentration. Interestingly, this minor boundary could be completely eliminated from the pattern by momentarily heating the detergent-protein solution to 75° C. Apparently, these differences in patterns of the solubilized protein fraction reflect degrees of molecular cleavage resulting from the action of the solubilizing agents or, as in the case of the detergent treatment, a binding of the detergent molecule with the protein, as suggested by Hill and Briggs *(5)*. A continued investigation of the action of detergents and alkaline sulfides on the insoluble protein fraction is in progress. The electrophoretic mobilities and corresponding areas of the migrating boundaries of the solubilized fraction shown in Figures 2 and 3 are reported (in Table 1).

Although the preparation of a dry sample by lyophilization had no apparent effect on the physical or electrophoretic characteristics of the protein in the soluble fraction, a similar treatment of a water suspension of the insoluble fraction was accompanied by a change in physical properties which rendered the protein partially unavailable to the solubilizing agents. Hence, for studies involving the insoluble protein fraction of the fat-globule membrane proteins(s) only freshly prepared suspensions were employed. Electrophoretic mobility patterns of the membrane protein(s) reported by Brunner *et al. (2)* were qualitatively similar to the patterns reported herein for the soluble fraction. They encountered cloudi-

TABLE 1

Electrophoretic mobility and relative percentages area of the migrating boundaries of the soluble and solubilized insoluble fat-globule membrane protein fractions, as determined from the average of ascending- and descending-channel electrophoretic mobility patterns shown in Figures 1, 2, and 3

Electrophoretic mobility patterns	Buffer system ($\Gamma/2 = 0.1$)	Migrating boundary [a]					
		1		2		3	
		u [b]	A %	u	A %	u	A %
Figure 1	Soluble membrane protein fraction						
Row 1	Glycine-HCl, pH [c] 2.05	+5.50	40.4	+4.16	48.9	+2.07	10.7
2	Acetate, pH 5.30	−0.95	48.0	−0.54	51.7	.....	.....
3	Phosphate, pH 6.10	−3.21	11.0	−2.30	63.8	−1.21	25.2
4	Veronal, pH 8.70	−5.22	38.2	−4.52	55.9	−2.81	6.0
Figure 2	Na_2 S-Solubilized insoluble membrane protein fraction						
Row 1	Phosphate, pH 6.10	−3.88	100.1	.....	.....	.....	.....
2	Phosphate, pH 7.30	−6.81	3.5	−6.01	96.5	.....	.....
3	Veronal, pH 8.70	−6.11	16.1	−5.75	83.9	.....	.....
4	Veronal, pH 8.70	−9.15	8.1	−7.12	91.9	.....	.....
Figure 3	Detergent-Solubilized insoluble membrane protein fraction						
Row 1	Veronal, pH 8.70	−9.80	32.5	−9.33	52.9	−8.10	14.6
2	Veronal, pH 8.70	−9.52	32.4	−7.85	67.6	.....	.....
3	Veronal-NaCl, pH 8.50	−11.63	11.8	−9.05	83.6	−6.79	4.6
4	Veronal, pH 8.70	−10.20	41.7	−8.82	48.7	−6.87	9.6

[a] Designation of boundaries shown in Figures 1, 2, and 3.
[b] Mobility (u) = cm.^{2}, sec.$^{-1}$, v.$^{-1}$, $\times 10^{-5}$; migrating distances measured from initial boundary.
[c] pH values extrapolated to 1° C.

ness in protein-buffer solutions prepared for electrophoretic analysis, which was largely removed by centrifugation and was believed to be due to the presence of uncleaved lipoproteins. Results from the present study suggest that the cloudiness should have been attributed to the presence of a suspension of an insoluble protein fraction, heretofore unrecognized in the early work in this field.

The solubilized protein fraction in all probability represents a denatured form of the native membrane protein moiety, therefore rendering absolute characterization somewhat speculative. Nevertheless, these data do contribute to our general knowledge of the protein. Zein, the insoluble protein of corn, has been solubilized and characterized in a similar manner *(3)*.

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A CHROMATOGRAPHIC TECHNIQUE FOR THE ESTIMATION OF SOME OF THE FREE FATTY ACIDS IN LIPOLYZED DAIRY PRODUCTS

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SUMMARY

A simplified Ramsey-Patterson partition chromatographic method is described for use on short-chain fatty acids (butyric through capric) normally found in dairy products. Procedures are described for preparing samples for analysis on a prepared column. The accuracy of the method is illustrated by a chromatogram of a known mixture of synthetic acids and by an analysis of saponified butteroil, in comparison with analyses of other investigators. An indication of the precision is given by five analyses of the same sample. Typical chromatograms are tabulated and explained.

Several methods *(1, 3, 4, 5, 7)* have been proposed for separating various fatty acids by chromatography. Ramsey and Patterson *(6)* introduced a chromatographic technique in 1948 for separating fatty acids of the group valeric through capric. Application of the method is restricted to samples dispersible in isooctane, in the absence of organic impurities that could disturb the partition rate.

Ramsey and Patterson proposed their method for the odd- and even-numbered carbon atom straight-chain fatty acids C5 through C10. Odd-numbered carbon atom fatty acids have not been reported in the fatty acids extracted from rancid milk products. Column characteristics of the Ramsey and Patterson method can be modified because of the absence of odd-numbered carbon atom fatty acids, so that development is more rapid. Faster development permits extending the method to butyric acid. A modification of the Ramsey and Patterson method is presented which permits chromatographic separation of butyric, caproic, caprylic, capric, and lauric plus higher fatty acids. A preliminary transfer is necessary into isooctane from an organic extraction medium used to extract the total fatty acids from lipolyzed milk products and related materials.

In general, the modification of the method is as follows: Milk product, silicic acid, and water are ground together in a mortar to yield a homogeneous, free-flowing, dustless, granular product. This product is quantitatively transferred to a tube and extracted with solvent into a flask. Isooctane is added to the effluent and the extracting solvent removed by fractional distillation. An aliquot of the residue is deposited on a prepared column and developed through butyric acid.

The prepared column consists of silicic acid, bromocresol green indicator, methanol, and ammonium hydroxide combined by a procedure that is readily reproducible.

EXPERIMENTAL PROCEDURE

Reagents.

Silicic acid. Mallinckrodt No. 2847, 100-mesh, suitable for chromatographic analysis by the method of Ramsey and Patterson.

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Internal phase. Make 100 mg. bromocresol green and 0.25 – 0.50 ml. concentrated reagent ammonium hydroxide to 250 ml. with absolute methanol. A 15-ml. sample of this blue internal phase should titrate to the first orange color with 3.5 ± 0.4 ml. of 0.10 *N* aqueous standard hydrochloric acid. If not in this range, adjust with additions of ammonium hydroxide or absolute methanol. The concentration of the indicator, bromocresol green, is not critical. Do not use internal phase that is over 1 mo. old.

Outside phase. Isooctane saturated with 90% methanol by shaking one liter isooctane with 20 ml. 90% methanol. Allow the phases to separate, and discard the lower methanol layer.

Isooctane. 2,2,4–Trimethylpentane. A good technical grade such as Phillips Petroleum Company commercial is satisfactory. Isooctane is usually acid-free; if not, wash with aqueous alkali, dry, and distill.

Extracting solution or solvent. 50% Acetone in chloroform by volume (AC).

Indicator. Dissolve 1 g. phenolphthalein in 50 ml. 95% ethanol and make to 100 ml. with isopropanol.

Standard alkali. Saturate isopropanol with potassium hydroxide by the procedure described by Keeney *(2)*. In this preparation add 25 g. 85% potassium hydroxide pellets to 400 ml. isopropanol. Dissolve by warming. Let the aqueous fraction settle. Pour off the isopropanol layer, stopper, and store in a refrigerator. To make the 0.015 *N* standard, dilute a suitable portion, such as 5 ml., to 500 ml. with 245 ml. isopropanol and methanol. Store in an amber glass bottle away from light when not in use. Standardize against a primary standard daily when in use.

Primary standard benzoic acid. A suitable solution is to make a known amount of benzoic acid, such as 5 g., to one liter with 500 ml. absolute methanol and isopropanol. This reagent will maintain normality for several months if stored under 0° or lower temperatures.

Special equipment.

Chromatographic tube. Corning 38450 tubes, 20 by 400, with coarse fritted disk.

Fractional still. Vigreaux tube, 470 mm., fitted between a 300-ml. boiling flask and condenser. Condensate flask cooled in ice water.

Extraction tube. A shortened chromatographic tube or suitable sized Buchner funnel with fritted disk.

Product preparatory equipment. Evaporation dish and pestle, powder funnel, torsion balance, etc.

Column forming equipment. Powder funnel, spiral wire, glass rod, glass-stoppered 125-ml. Erlenmeyer flask, cork plunger, etc.

Pressure regulating equipment. Compressed air led into the column with plastic and glass tubing. Include bleeder branch with pinch clamp to regulate pressure by controlling the bleeder escape rate.

Extraction of fatty acids from a lipolyzed milk powder.

Weigh into an evaporating dish 4 g. of silicic acid. Add 2 g. of rancid milk powder. Add 1 ml. water. Mix with the pestle until a granular, homogeneous,

dustless mass is obtained. If sticky, too much water has been added. If material sticks to the pestle or dish, a spatula is used to free the material and grinding is continued. High-fat products may require proportionately more silicic acid and less water. When mixing is judged to be complete, transfer into the fritted funnel or tube with the aid of a powder funnel and spatula. This transfer of material will not be quantitative. After a filter paper circle has been pressed down on the material in the tube, quantitatively rinse the powder funnel, spatula, and pestle into the dish. Then transfer the rinsings from the dish into the tube. Not more than 15 ml. AC in 4-ml. increments should be needed for rinsing. Force the rinsings just into the column with pressure, add 5 ml. extracting solvent to rinse down the tube, force this into the column, then finish the extraction by forcing through an effluent volume of 60 ml. extracting solvent. Collection should be in a flask suitable for use as a boiling flask in the fractional still.

Butyric acid added to milk powder extracts more slowly than the longer chain fatty acids. Tests indicate that added butyric is extracted when 40 ml. effluent has been collected; 60 ml. is collected to insure complete extraction. Table 4 acid degree values for the powder check within $\pm$ 1.1% on five extractions—an indication that extraction is complete.

The extraction procedure has been applied to butteroil, and to the following lipolyzed products: dry whole milk, dry whey, fluid milk and cream, cheese products, milk chocolate, butterfat emulsions, and animal tissue extracts. Sample size is adjusted to supply an aliquot to deposit on the column that will require at least 8 ml. 0.015 *N* alkali to neutralize. Adjust the quantity of silicic acid and water to give a dustless, granular, free-flowing mixture after blending.

Add 14 ml. isooctane to the flask containing the extracted AC mixture. Start the fractional distillation and continue until distillation ceases with the column at 74° C. Residue volume should be less than 10 ml. Allow flask and residue to air-cool, then disconnect the flask and stopper. Place the distillate collection vessel below the Vigreaux column. Rinse the column with 5 ml. isooctane collecting in the distillate flask. Titrate the flask of distillate etc., to the phenolphthalein endpoint with standard alkali. A blank should be deducted. The blank is determined by titrating the distillate from a blank consisting of the fractional distillation of 60 ml. AC and 15 ml. isooctane. This blank will be about 0.20 ml. of 0.01 *N* alkali. As in all fractional distillations, bumping will cause error because the equilibrium of the column is disturbed; glass beads may be used to prevent bumping. Any net tritration in the condensate is butyric acid if only C4 and higher fatty acids are present in the sample. However, acetic and propionic acid, if present, will also partially distill over.

Preparation of the chromatographic column.

Weigh 16 g. silicic acid into a 125-ml. glass-stoppered Erlenmeyer flask. Add 12 ml. of internal phase. Cool in ice water or cold tap water for 5 min. Shake until all solid material is free of the glass of the flask. This requires 5–10 min. of vigorous, intermittent shaking; pressure which may build up during this shaking from internal heating should be released. If the final homogeneous powder is dusty, more internal phase is needed and should be added in 0.5-ml.

increments until a free-flowing, dustless powder results. If the quantity of internal phase used leaves the finished mixture sticky, 0.5 ml. less internal phase should be used in subsequent preparations until the finished powder is no longer sticky. Allow freshly prepared packing material to stand overnight at room temperature before using.

Clamp a chromatographic tube in a vertical position; connect a short glass tube to the bottom with tygon tubing. Use a screw clamp to pinch off the tygon tubing. Place a circle of filter paper on the fritted disk of the tube. Add 30 ml. developing solution to the tube. Place a powder funnel in the top of the tube and pour in the stationary phase. The transfer need be qualitative only. Insert the glass rod and swirl the stationary phase, breaking up any lumps against the wall of the tube. Remove the rod, insert the wire spiral, and with suitable spinning plunger action dispel all air bubbles and evenly disperse the stationary phase. Remove the spiral, rinse down the sides of the tube with developing solution, open bottom clamp, and apply pressure to pack the stationary phase and drain excess developing solution. With the level of developing solution still above the packed column, insert a second filter paper disk and press firmly against the stationary phase with the cork plunger. Just before adding the sample, drain the developing solution to the top of the packed column.

Analysis of fatty acids on the column.

When the sample is ready in isooctane, and the column has been prepared, remove the tygon tubing and clamp from the bottom of the tube. Add the sample in 1- or 2-ml. increments, with a sum of not more than 4 ml., to the top of the column. Apply pressure to drain the last addition just to the top of the column, then rinse down the walls of the tube etc., with three successive 1.5-ml. portions of the developing solution, draining each portion just into the column before the next is added. Then fill the tube with developing solution, again apply pressure, and continue as long as acidity is detected by titrating the effluent. From the time sample is added until the chromatogram is complete, the top of the column must not be allowed to run dry.

Start collecting effluent in graduated containers as soon as the first increment of sample is added to the column. The fraction volumes should be collected as listed in Table 3 if no previous data on the samples are available. By titrating each fraction, then calculating the effluent acidity per milliliter of effluent, and by observing the movement of the yellow bands, the various fractions that can be obtained are, in order, lauric and higher acids, capric, caprylic, caproic, and butyric acid. Other acids, if present, will remain on the column as stationary or slowly moving bands. Propionic acid which is present in some rancid dairy products develops off the column immediately after butyric. When analyzing samples that contain propionic acid, the blank value can not be determined by titration until the propionic acid is eluted. Acetic acid follows propionic, but its threshold is sufficiently far behind propionic to permit a true blank to be obtained.

To check the identity of any band that may be in question, a known acid may be added to the column with the sample, or a synthetic sample containing known fatty acids may be separated, using a duplicate column and reagents. Recoveries

of added acids and contamination by higher and lower homologs were checked by Ramsey and Patterson *(6)*. Separation was excellent; contamination is likely only by the next higher homolog.

A fresh column must be prepared for each chromatogram. The amount of silicic acid is always 16 g. for each chromatogram. Some supplies of silicic acid are unsatisfactory and will not separate the fatty acids when this quantity is used. Any silicic acid unsatisfactory for chromatographic columns can be used in the extraction procedure, where its purpose is to increase surface area for contact between extracting solvent and sample.

RESULTS

Calculation of molar percentages of each fatty acid, and of total acidity from typical chromatograms.

Table 1 contains the tabulated data from a chromatogram of a sample of

TABLE 1

Chromatogram of saponified butteroil

Effluent Fraction No.	Volume		Titration	Blank	Net	Acid		Hilditch[a]
						(amount)	*(mole %)*	*(mole %)*
1	10		0.05	0.05	0			
2	12		15.25	0.05	15.20		Lauric and higher	
3	15		1.44	0.06	1.38	16.65	79.2	82.1
4	2	39	0.08	0.01	0.07			
5	2		0.18	0.01	0.17			
6	4		0.36	0.02	0.34		Capric	
7	12		0.27	0.05	0.22	0.75	3.6	3.1
8	4	22	0.04	0.02	0.02			
9	4		0.06	0.02	0.04			
10	4		0.08	0.02	0.06		Caprylic	
11	20		0.46	0.07	0.39	0.51	2.4	1.9
12	20		0.09	0.07	0.02			
13	10	58	0.04	0.04	0			
14	30		0.66	0.09	0.57			
15	30		0.45	0.09	0.36		Caproic	
16	30		0.14	0.09	0.05	0.99	4.7	3.4
17	30	120	0.10	0.09	0.01			
18	30		0.37	0.09	0.28			
19	30		1.19	0.09	1.10		Butyric	
20	30		0.58	0.09	0.49	2.12	10.1	9.5
21	30		0.27	0.09	0.18			
22	30		0.15	0.09	0.06			
23	10	160	0.04	0.03	0.01			
	399		22.35	1.33	21.02			

Blank determination: Net titration was 21.40 ml. of alkali for an aliquot equal to the free fatty acid sample deposited on the column. The sum of titrations of effluent fractions off the column was 22.35 ml. By difference the blank value is 0.95 ml. (22.35 minus 21.40 equal 0.95). Column effluent fractions that might be blank values are Numbers 1, 4, 8, 13, 17, and 23. Visual inspection eliminates Numbers 4 and 8. The other four average 0.0041 for 1 ml. effluent. For 399 ml. effluent this would give a blank of 1.64 ml. The two methods for obtaining a blank value average 1.30 ml. This was used as the blank value in calculating individual fraction blanks which yield a net blank of 1.33 ml. Flow rate of the developing solution was increased toward the end of the run, which results in a slight decrease in blank titration for the last nine fractions.

[a] Values in this column are tabulated from results reported on page 126 of *The Chemical Constitution of Natural Fats*, by Hilditch, 3rd ed., published by John Wiley and Sons, Inc. in 1956.

saponified butteroil. The butteroil used in this chromatogram was saponified and taken up in isooctane by a procedure similar to that described by Keeney *(2)*, except that isooctane replaced the hexane to extract the regenerated fatty acids. This table demonstrated the typical cutting of fractions, so that acid minima are obtained to separate the various component acid bands, even when the bands are invisible. Blank values are obtained by titrating effluent fractions from the column that are known to be acid-free, or by calculation of difference between total acidity off the column and net acidity of a sample of the same size as deposited on the column. Both methods should give essentially the same blank value, unless there is acidity in the sample that is not eluted from the column. The note following Table 1 compares these values. The last column gives typical molar percentages of fatty acids found in milk fat from New Zealand and England, as reported by Hilditch. Keeney *(2)* reports an average value of 10.4% butyric acid obtained from analyses of 500 lots of butterfat.

TABLE 2

Chromatogram of synthetic fatty acid mixture

Effluent Fractions	Volume	Net titration	Mole %	Acid	Calc. mole %
4	41 ml.	8.39 ml.	46.9	Lauric etc.	45.2
6	20	0.77	4.3	Capric	4.9
5	39	0.75	4.2	Caprylic	4.1
5	100	1.87	10.4	Caproic	10.4
5	140	6.11	34.2	Butyric	35.4
1	10	0	.....		.....
26	350	17.89	100.0		100.0

The 350 ml. effluent titrated 19.29 ml. The blank calculated from the titration of acid-free effluent fractions times effluent volume was 1.40 ml., leaving a net titration off the column of 17.89 ml., recorded above. Direct titration of a duplicate quantity of sample that was chromatographed was 17.75 ml. net. The calculated titration of the sample chromatographed was 17.80 ml. The lauric etc., fraction contained lauric, palmitic, stearic, and oleic acids.

Table 2 contains the data for a chromatogram of a synthetic fatty acid mixture. All acids were visible as yellow bands moving down the column. Each portion of effluent containing an individual fatty acid was collected as a single fraction. A small fraction was taken between each acid fraction, to check the visual separation of the fatty acids. Only the fraction between lauric etc., and capric acid contained any acidity; this was placed in the lauric etc., group. The fatty acids were identified by threshold volume and the known composition of the mixture.

Table 3 is the tabulated data for a typical analysis of a commercial rancid milk powder marketed under the trade name Mil-Lait. At the bottom of the table is an explanation of the calculation of total acidity as the acid degree value and the explanation of how the various values are obtained that are used in calculating the mole per cent acidities.

Rancid dry whole milk, Mil-Lait, with and without added butyric acid, was extracted and chromatographed; butyric acid recoveries were 97–104%.

Table 4 indicates the variations that one can expect in analyses of a sample.

TABLE 3

Chromatogram of a commercial lipolyzed whole milk powder called "Mil-Lait"

Effluent Fraction No.	Volume	Titration	Blank	Net	Acid Amount	Mole %
1	10	0.06	0.06	0		
2	15	3.50	0.09	3.41	Lauric and higher	
3	12	2.70	0.08	2.62	6.34	44.2
4	3	0.20	0.02	0.18		
5	3	0.15	0.02	0.13		
6	3	0.20	0.02	0.18		
7	5	0.41	0.03	0.38	Capric	
8	4	0.20	0.03	0.17	0.89	6.2
9	4	0.11	0.03	0.08		
10	4	0.07	0.03	0.04		
11	4	0.07	0.03	0.04		
12	10	0.33	0.06	0.27		
13	10	0.26	0.06	0.20	Caprylic	
14	10	0.11	0.06	0.05	0.52	3.6
15	10	0.06	0.06	0		
16	20	0.56	0.12	0.44		
17	20	0.91	0.12	0.79	Caproic	
18	20	0.23	0.12	0.11	1.34	9.3
19	20	0.12	0.12	0		
20	20	0.31	0.12	0.19		
21	30	3.67	0.18	3.49	Butyric	
22	30	1.40	0.18	1.22	5.27	36.7
23	20	0.27	0.12	0.15		
24	20	0.13	0.12	0.01		
	307	16.03	1.88	14.15		
			Condensate	.21		
				14.36	14.36	100

Standard alkali 0.0120 *N*. A 3-ml. sample was deposited; a duplicate titrated 14.1 ml. net. A 4-g. sample was taken containing 1.14 g. fat. Net titration of the condensate from the fractional distillation was 0.6 ml. After the two 3-ml. aliquots were removed from the residue in the flask, the remainder titrated 12.55 ml. Sample acidity was, therefore, 41.35 ml. of 0.012 *N* alkali. The acid degree value equals $(1.0/1.14)\times(.012/.01)$ times 41.35, or 43.5. In the calculation of mole per cent fatty acids, the condensate acidity is calculated as butyric acid; the quantity equivalent to the sample deposited on the column is $0.6\times(14.1/40.75)=0.21$.

TABLE 4

Mole per cent fatty acids of five separate analyses of a commercial lot of "Mil-Lait" made December 5, 1956

Fatty acid	Analyzed 3/5/57	3/6	3/12	3/13a	3/13b	Av.
			(%)			
Lauric etc.	44.3	44.6	44.1	43.2	43.7	44.0
Capric	5.4	5.9	6.8	6.5	6.9	6.3
Caprylic	3.7	3.5	4.1	4.1	4.3	3.9
Caproic	8.8	9.1	8.9	9.3	9.4	9.1
Butyric	37.8	36.9	36.1	36.9	35.7	36.7
A.D.V. Powder	42.3	42.8	43.2	42.8	42.9	42.8
A.D.V. of sample deposited	14.8	15.6	11.6	11.4	12.7	

The products analyzed in this table and in Table 3 are similar but manufactured 4 mo. apart. A comparison of molar percentages of individual fatty acids will give an example of variations between similar products.

A.D.V., acid degree value, is the milliliters of 0.01 *N* alkali required to neutralize the acidity of 1 g. of fat.

Capric acid varies much more than any other component, because this acid follows lauric etc., acids very closely. Therefore, if the chromatogram does not develop with a uniform front, the titration minima between capric acid and the higher homologs may be obscure.

Threshold volumes of the lauric and higher component and of the capric acid component are fairly independent of chromatogram variable. Threshold volumes of the other components, however, should be determined whenever a new ingredient is used in the preparation or development of the chromatogram. These threshold volumes should be obtained on a synthetic sample mixture containing sufficient caprylic, caproic, and butyric acid for visual cutting of fractions.

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A GAS-LIQUID CHROMATOGRAPHIC METHOD FOR VOLATILE FATTY ACIDS IN MILK[1, 2]

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SUMMARY

Gas-liquid partition chromatography provided a means for a rapid and accurate analysis for the volatile fatty acids in milk. The chromatographic apparatus utilized a 6-mm. I.D. glass column 4 ft. long and packed with Celite 545 containing 25% liquid phase. The liquid phase consisted of silicone-stearic acid (95/5 w/w) mixture. The operating temperature of the column was controlled by use of a heating jacket through which glycerol could be circulated.

Analysis for formic, acetic, propionic, butyric, valeric, caproic, and caprylic acids could be completed in 90 min. This was achieved using a constant nitrogen gas pressure of 100 cm. of mercury and progressively increasing the column temperature from 60–160° C. Quantitative recovery of a mixture of the seven pure acids was obtained in all trials.

The water soluble–ether extractable acids were obtained from milk, using a modified Roese-Gottlieb procedure on acid filtrates. Recoveries of acids added to milk varied for each acid investigated. Recoveries of formic, acetic, and butyric acids were 57%, 86%, and 81%, respectively. Propionic, valeric, caproic, and caprylic acids were recovered quantitatively. Replicate analyses revealed that the extraction procedure provided results reproducible within ± 5%.

Gas-liquid partition chromatography is an extension of the theory and principles of gas chromatography to apply to relatively volatile liquids. Phillips *(6)* has reviewed the theory and applications of this technique for the separation of various organic compounds. James and Martin *(2)* introduced this procedure in 1952 for the separation and micro-estimation of volatile fatty acids, and for the methyl esters of fatty acids *(3)*. Certain modifications in the James and Martin procedure for fatty acids were made by Van de Kamer *et al. (7)* to improve the separation and yields of volatile fatty acids from formic through dodecanoic in one-column analysis. Gas-liquid chromatography was used qualitatively by Jennings *(4)* to study the volatile compounds in starter culture distillate.

Preliminary attempts in our laboratory to employ the gas-liquid chromatographic method of Van de Kamer *et al. (7, 8)* resulted in unsatisfactory yields and incomplete resolution of some acids. Consequently, work was undertaken to develop a reliable method. After this study was completed, Hawke *(1)* reported the use of a different liquid phase for the volatile acids. He applied this method to quantitative analysis of volatile fatty acids in milk fat and applied gas-liquid chromatography of methyl esters for the analysis of non-volatile fatty acids in milk fat.

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The work reported in this paper had as its objective the development of a simple, rapid, and accurate gas-liquid chromatographic method to measure the water-soluble, volatile free fatty acids in milk and dairy products.

EXPERIMENTAL PROCEDURE

Extraction procedure for volatile acids from milk. A prerequisite to the chromatographic procedure was the development of a satisfactory extraction procedure to obtain volatile acids from milk in good yield and in concentration suitable for chromatography. A modified Roese-Gottlieb extraction procedure was found to give better results than either a butanol or a chloroform-butanol extraction method.

The procedure finally adopted was as follows: *(a)* Five ml. 5% (V/V) sulfuric acid was added to 100 ml. of milk and the mixture filtered through Whatman No. 1 filter paper, *(b)* to 50 ml. of the filtrate in a separatory funnel was added 5 ml. of 5% (by volume) sulfuric acid, 50 ml. of ethanol, 100 ml. ethyl ether, and 100 ml. petroleum ether, *(c)* the mixture was shaken ten times after the addition of each of the first two reagents, and 40 times after the addition of each of the latter two reagents, *(d)* the extraction was repeated twice, using the same volumes of ethyl and petroleum ether but no alcohol, *(e)* the three ether extracts were combined in a beaker, 0.5 ml. alcoholic phenol red indicator was added, and the mixture neutralized with 1.0 ml. 1 *N* alcoholic sodium hydroxide, *(f)* the sodium salts were evaporated to dryness at room temperature, *(g)* the dried salts were transferred to a 50-ml. Erlenmeyer flask acidified with 0.2 ml. 5 *N* HCl, made to a specific volume with ether, and an excess of anhydrous sodium sulfate added, *(h)* the ether solution was kept in tightly stoppered flasks and stored at 4° C. Proper acidification and neutralization in the extraction and concentration procedure were very important in preventing loss of acids.

Chromatography apparatus. The apparatus assembled, which was basically that used by James and Martin *(1)* as modified by Van de Kamer *et al. (6)*, is illustrated (Figure 1). The chromatographic tube containing the packing

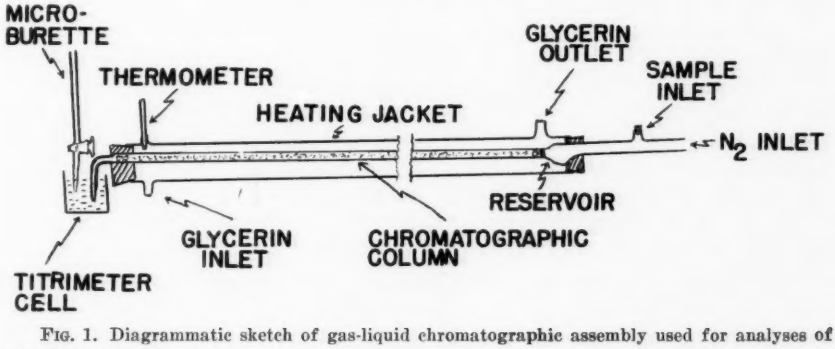

FIG. 1. Diagrammatic sketch of gas-liquid chromatographic assembly used for analyses of volatile fatty acids.

material was a 4-ft.-long, 6-mm. I.D. glass tube joined at the outlet ends to a 3-in. piece of heavy-walled glass tubing, and at the inlet end to a 1-ft. length of 12-mm. glass capillary tubing with a 5-ml. capacity reservoir at the juncture.

The inlet end of the column had two openings, one for the nitrogen gas used as a carrier and the other for introduction of the sample to be analyzed. The nitrogen gas (dry grade) was first passed through a $CaCl_2$ drying chamber and the pressure regulated, using a mercury manometer. The outlet end of the column protruded only far enough from the heating jacket to permit the attachment of a short right-angled glass delivery tip, by means of a rubber connection, to discharge into the titration vessel of a Beckman titrimeter. A short outlet end was important to prevent condensation of the higher acids.

The entire glass column, except for the outlet and inlet ends, was sealed into the brass heating jacket with rubber stoppers. Glycerin, which was used as the heating medium, was recirculated through the brass heating jacket. An electric thermostat in the heating bath permitted careful regulation and duplication of the heating rate over a range from 60 to 160° C.

Preparation of the column. The column employed Celite 545 [4] as a support, silicone and stearic acid as the liquid immobile phase, and nitrogen gas as the eluting gas. The Celite was sized by washing and retaining the coarser granular material. The Celite particles, which settled 18 cm. in 2 min. on repeated washings with water, were heated for 3 hr. at 300° C., washed with concentrated HCl, washed free of acid with distilled water, and dried at 150° C.

Dow Corning 550 Silicone[5] with 5% added stearic acid (W/W) was found to be the most satisfactory immobile liquid phase.

A mixture of two parts Celite to one part of liquid phase as recommended by previous workers *(1)* and *(6)* was difficult to pack uniformly and resulted in unsatisfactory resolutions of the fatty acids. When three parts of Celite were added to one part of liquid phase, the column packed better and superior analyses were obtained. To provide for a more uniform column material, the liquid phase was dissolved in an excess of ethyl ether, to which the Celite was added with stirring to form a slurry. The ether was evaporated on a steam bath with occasional stirring to give a homogenous mixture.

Packing of the column. A piece of glass wool was inserted at the inlet end of the column and seated against the outlet capillary tip, which was attached to a vacuum pump. The column was vibrated continuously with a vibrator while the packing material was being fed into the open end, to obtain a uniformly packed column. The column was filled with material to the edge of the sample reservoir and a second glass wool plug was seated against the compacted material. After the column was assembled it was sealed to avoid getting glycerin in the column tip when placing the column in the heating jacket. The tip was kept sealed when not in use, to avoid absorption of moisture.

Chromatographic procedure. Temperature, pressure, and time variables were

[4] Marketed by Johns-Manville Corp., New York, N.Y.

[5] Manufactured by Dow Corning Corporation, Midland, Michigan.

investigated over a broad range to determine the optimum operating conditions for the separation of formic, acetic, propionic, butyric, valeric, caproic, and caprylic acids.

The most satisfactory procedure utilized a constant nitrogen pressure of 100 cm. The temperature of the column at time of starting circulation of the glycerin was 60° C. and the temperature was increased to 160° C. during 60 min. In this manner optimum temperature for the elution of each acid was achieved. A typical time-temperature curve is shown (Figure 2).

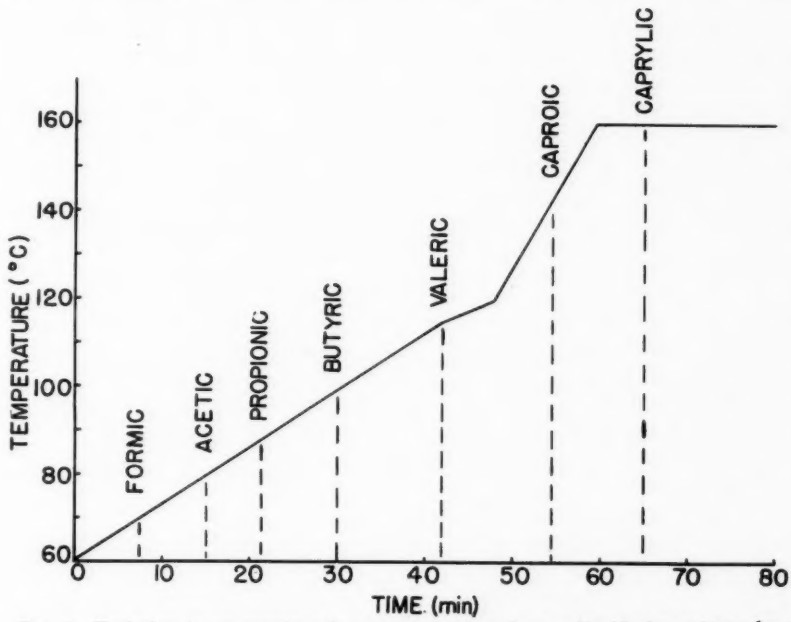

FIG. 2. Variation in temperature in respect to time for gas-liquid chromatography of volatile fatty acids.

The sample was added by using a syringe with a 3-in. hypodermic needle, to avoid volatilization. A 0.2-ml. aliquot of the ether was used to rinse any residual sample from the inlets. The inlet was sealed and nitrogen pressure applied. Nitrogen was passed through the column for 5 min. to volatilize the ether. After exactly 5 min. glycerol circulation was commenced. A stop watch was used for timing the run. The acids were collected in neutral distilled water and titrated to a phenol red end-point with 0.01 *N* sodium hydroxide by hand or with an automatic titrimeter as they were eluted. When a recorder was not used the titrations were made at 0.5 min. for 10 min. and then every minute thereafter. The temperature of the column was checked and recorded at similar intervals. At the end of 40 min. (120° C.) the column was heated rapidly to 160° C., and maintained at this temperature for the duration of the run (80 min.).

A standard curve for known acids was prepared as follows: approximately 0.5 ml. of each of the seven pure acids were diluted with ethyl ether to 50 ml. in glass-stoppered volumetric flasks and anhydrous Na_2SO_4 was added. A specific amount of dilution (10–100 m*M*) was added to the column. A new standard curve was established for each column.

RESULTS

Application to pure acids. A typical curve showing resolution of formic, acetic, propionic, butyric, valeric, caproic, and caprylic acid is in Figure 3. The resolution for formic through caproic was very good, but rather broad peaks with considerable "tailing" were observed for caprylic acid. Time for the initial elution of individual acids varied from 3 to 5 min., between four replicate trials with a similar variation in the termination of the elution (Table 1). However, the maximum variation in elution time was not sufficient to prevent the identification of any of the fatty acids in subsequent analyses of unknowns. Recoveries of known amounts of pure fatty acids were above 98% for formic, acetic, propionic, and butyric acids and between 90 to

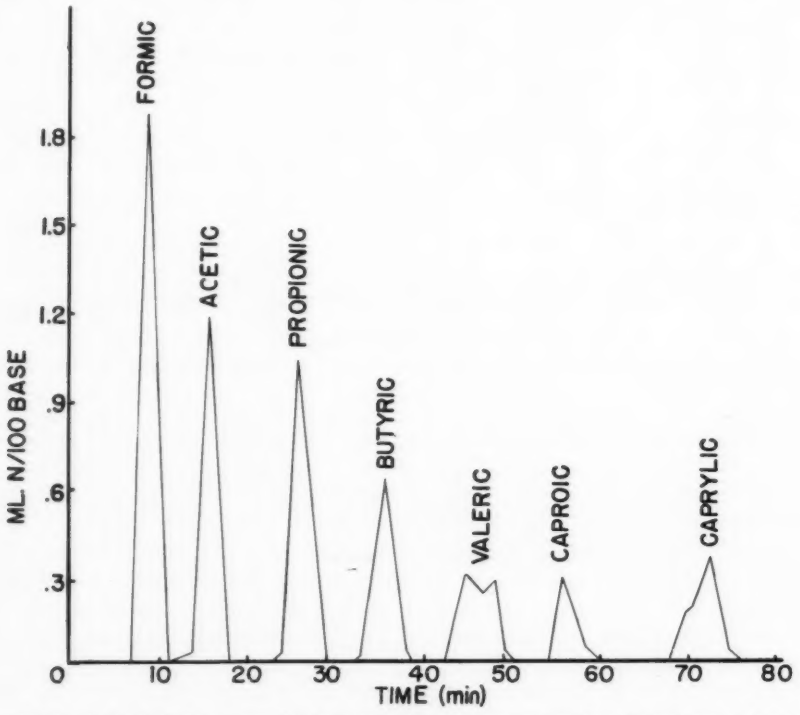

FIG. 3. Typical elution curve obtained in gas-liquid chromatography of mixtures of formic, acetic, propionic, butyric, valeric, caproic, and caprylic acids.

95% for valeric, caproic, and caprylic acids. The variations in the amounts of acid recovered did not exceed ± 5% in any of the four trials for any of the seven pure acids.

TABLE 1

Recovery of mixtures of pure acids by gas-liquid chromatography [a]

Acid	Range in elution time (min.)		μM acid Added	Recovery	
	Initiation	Termination		Range	Average
Formic	7–8	10–13	40	98.1–104.1	102
Acetic	10–15	14–19	30	99.0–103.7	101
Propionic	22–26	26–30	20	98.3–105.4	103
Butyric	32–35	35–37	38	94.2–101.3	98.7
Valeric	40–45	47–51	32	88.7– 96.3	90
Caproic	53–57	58–61	30	92.7– 99.3	95.6
Caprylic	65–69	71–74	31	94.7– 99.6	96.8

[a] Data based on four trials.

Application to measurement of free fatty acids in milk. A typical elution curve for free volatile fatty acids extracted from fresh milk is shown (Figure 4). Identification indicated in the figure was based on the elution time of each component and the attainment of identical elution time when pure known acids were added to the sample at the time of chromatography.

Known concentrations of pure acids were added to the milk filtrates and subjected to extraction, chromatography, and titration. The results of recovery of these acids are shown (Table 2). The results reveal that variations in recoveries were obtained with individual acids, with relatively low recoveries of formic (56.7%), butyric (81.4%), and acetic (86.5%). The variation in recovery in individual trials is slight, indicating good reproducibility of the method.

TABLE 2

Recovery of pure acids added to milk filtrates after extraction and gas-liquid chromatography

Acid	No. of trials	μM added	μM recovered		Average % recovery
			Range	Average	
Formic	4	40	22.5–22.9	22.7	56.7
Acetic	4	30	24.9–26.9	25.8	86.5
Propionic	3	20	19.0–20.2	19.6	98.0
Butyric	4	38	30.4–31.2	31.1	81.4
Valeric	3	33	31.6–32.6	32.1	96.4
Caproic	3	30	28.4–29.5	29.0	96.6
Caprylic	3	31	29.2–29.9	29.6	98.5

Further evidence of reproducibility was obtained by dividing a sample of milk into five lots, extracting and chromatographing the volatile free acids from each lot (Table 3). The results indicated average deviation of from 2.3 to 4.6% for the individual free fatty acids.

Determination of possible interference of certain nonvolatile acids. Certain acids known to exist in fresh milk, which presumably would be in the ether extract, were added to the gas-liquid column, to determine whether or not

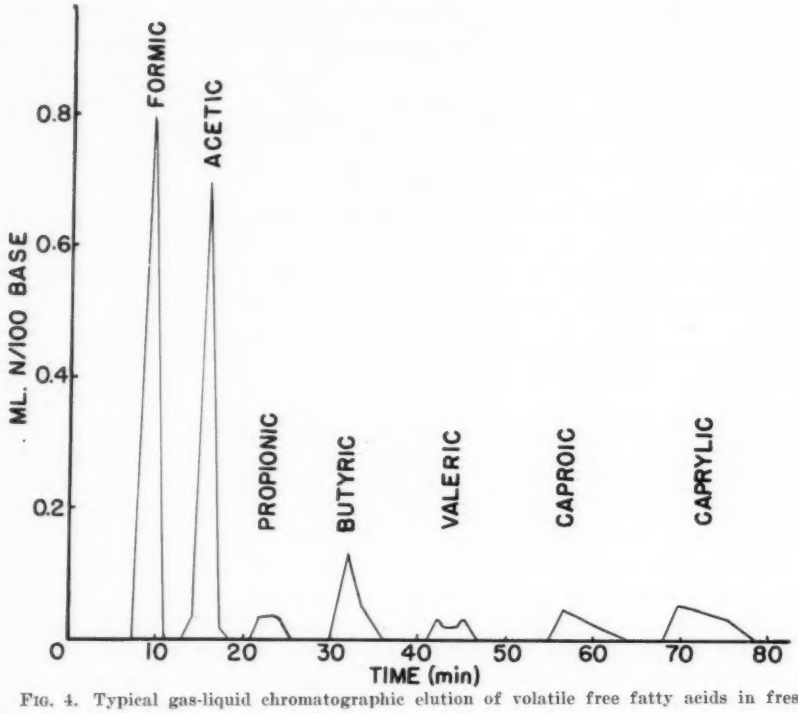

FIG. 4. Typical gas-liquid chromatographic elution of volatile free fatty acids in fresh milk.

TABLE 3

Reproducibility of volatile acid analyses of a fresh raw milk sample [a]

Acid	μM/100 ml. of filtrate		% Maximum deviation	% Average deviation
	Range	Average		
Formic	43.2–50.4	48.2	4.9	3.7
Acetic	20.7–22.1	21.3	5.2	2.3
Propionic	3.6– 3.85	3.1	4.6	3.2
Butyric	3.3– 3.6	3.4	5.5	4.4
Valeric	4.2– 4.5	4.4	4.8	3.4
Caproic	7.4– 7.9	7.6	4.3	3.3
Caprylic	9.2–10.1	9.7	5.1	4.6

[a] Average of four trials.

they might interfere with the analyses. These acids, lactic, alpha-ketoglutaric, pyruvic, and beta-hydroxybutyric did not volatilize under the operation conditions employed, but were retained on the column. They did not affect the separation of the volatile acids on subsequent trials. The only compound studied which was shown to volatilize was hydrogen sulfide, but this compound was eluted ahead of formic acid. Based upon these analyses, it would appear that the method probably is specific for the free volatile fatty acids.

DISCUSSION

Although the retention values of the compounds isolated from milk agree identically with those of known free fatty acids, the gas-liquid chromatographic method is subject to the basic limitation of all chromatographic procedures. Other volatile acidic compounds might have identical threshold retention values as the known compounds. However, the extraction procedure would exclude the majority of such compounds as possible interfering substances and the method appears specific for the volatile fatty acids.

The contrast in recovery of pure acids applied directly to the column, as compared to their recovery when added to milk filtrates, especially of formic, acetic, and butyric acids, reflects a loss which can be attributed to the concentration of the initial extracts. The recoveries from milk were of the same magnitude as reported by Morr *et al. (5)*, using a silica-gel partition chromatographic procedure. The loss of the formic and acetic acids was found to be related to the use of heat in the concentration of the alkaline salts of these acids.

In comparison to other gas-liquid methods *(1, 2, 7)* it would appear that the proposed procedure is more rapid and simpler in operation and equipment required. However, this method can not be readily utilized for analysis of fatty acids with a carbon chain of greater than eight carbon atoms.

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A RESAZURIN DISK ASSAY METHOD FOR DETECTING ANTIBIOTICS AND NATURAL STARTER INHIBITORY ACTIVITY IN MILK [1]

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SUMMARY

A relatively rapid method for detecting antibiotics in milk is described. It involves flushing the agar in the disk assay method with resazurin. The presence of antibiotics in milk is revealed by the retention of a red or purple zone in the plate due to the inhibition of the microbial reduction of resazurin by antibiotics. The method requires a few extra manipulations but takes less time than the standard disk assay test. It takes a total of 1.5–3.6 hr. The method can detect antibiotics and naturally existing inhibitory substances in milk.

To establish if the inhibition zones are due to antibiotics or to some other inherent antibacterial substance in milk, heating milk prior to testing, to inactivate the latter substances, may facilitate the differentiation. Heating milk also partially inactivates the antibiotics. The lowest concentrations that can be detected with the method are in the concentrations generally found to be capable of inhibiting starter activity. Normal residual concentrations of sanitizing agents can not be detected by this method.

Antibiotics used in the treatment of mastitis are secreted in milk and impede the lactic acid fermentation desired in the manufacture of cheese and other cultured products. During recent years, the incidence of antibiotics in milk has been the subject of considerable investigation. Welch *et al. (14)* conducted three nation-wide surveys and have reported that there has been an increase in the incidence of antibiotics in milk in 1955 and 1956 over 1954. The need is evident, therefore, for a relatively rapid method to determine the presence of antibiotics in raw milk intended for the manufacture of cultured dairy products.

There are available several chemical and bacteriological methods for the determination of antibiotic content of pharmaceutical preparations and biological materials. Previously, attempts were made in this laboratory to adapt to milk the colorimetric methods of Scudi *(9)* and Boxer and Everett *(1)*. These attempts were unsuccessful since these methods, originally devised for relatively high concentrations of antibiotics, could not be applied to the extremely small concentrations of antibiotics generally found in milk. Consideration was given to applying to milk the rapid fluorometric method developed by Scudi and Jelinek *(10)*. The method required the extraction of penicillin in an aqueous or chloroform solution. Preliminary trials made of this method were unsuccessful, since small concentrations of penicillin in milk could not be extracted quantitatively. Also, since the method is specific only for penicillin, it would not be applicable to other antibiotics.

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Among the bacteriological methods used during recent years, the acid inhibition method *(3)*, the disk assay method *(12)*, methylene blue and resazurin dye reduction methods *(2, 8, 13)*, alphanaphthylamine hydrochloride method *(4)*, and triphenyltetrazolium chloride method *(5)* are the more important ones. However, the acid inhibition method, the disk assay method, and the methylene blue method are time-consuming, taking 4–16 hr., and the resazurin and alphanaphthylamine hydrochloride methods are insensitive to low concentrations of antibiotics. All of these methods are, therefore, not quite satisfactory from the standpoint of the plant operator.

As a screening test for the selection of an antibiotic of choice for the treatment of disease, Pital *et al. (7)* have developed a simple and rapid qualitative method based upon the inhibition of microbial reduction of resazurin by antibiotics.

The objective of the present study was to develop a relatively rapid and sensitive method involving inhibition of a test culture by antibiotics in milk, using resazurin as an indicator.

MATERIALS AND METHODS

The method described herein essentially is a modification of the technique described by Pital *et al. (7)*. It involves performing the standard disk assay method and flushing the agar used in the method with resazurin. The antibiotic from milk in the disk diffuses into the agar, inhibits the test organism, and consequently retards the reduction of resazurin. Several modifications were introduced in the original method, in order to apply it to milk and to reduce the time required for the completion of the test.

Test organism. To select a test organism which could reduce resazurin in the shortest period of time, preliminary trials were conducted with eight different organisms sensitive to the common antibiotics used in mastitis therapy. They were: *Lactobacillus bulgaricus, Staphylococcus aureus, Streptococcus thermophilus, Streptococcus lactis,* a mixed commercial lactic culture, *Bacillus subtilis, Bacillus cereus,* and *Klebsiella pneumoniae. S. aureus* and *L. bulgaricus* were selected for further study. The remaining bacteria were discarded either because they took excessive time to complete the test or were found to be not very sensitive to small concentrations of the antibiotics used. To avoid daily transferring of the broth cultures, trials were made using spore suspensions of *B. subtilis* and *B. cereus.* It was also observed that spore suspensions took a longer time to complete the test than did the fresh broth suspensions of the organisms.

The stock cultures of the test organisms, *S. aureus* and *L. bulgaricus,* were grown and maintained on agar slants. For the experimental work, the organisms were transferred into tubes of penassay broth and tomato juice broth, respectively, and incubated at 37° C. for 14–15 hr. The broth cultures were transferred daily in fresh broth and were transferred for at least four times before being used.

Assay agar. Brain-heart infusion agar was used in the test employing *S. aureus,* and whey agar containing 10% filtered tomato juice was employed in the test using *L. bulgaricus.*

Resazurin solution. One hundred milliliters of distilled water were brought to a quick boil in a narrow-mouthed flask and six resazurin tablets (14 mg. each), previously powdered, were dissolved into it. The solution was stirred momentarily, cooled, and filtered through glass wool. The resazurin solution was prepared fresh every week and was stored in a brown bottle at room temperature.

Antibiotics. The antibiotics used were: penicillin, streptomycin, aureomycin, terramycin, and achromycin.

Procedure of the test. One hundred milliliters of the melted agar were inoculated with 4 ml. of 14-hr.-old broth culture of a test organism. Twenty milliliters of the inoculated agar were pipetted into a flat-bottomed Petri dish (Corning #3162). After the agar had solidified, a half-inch filter paper disk (S and S 470-E), soaked in the test milk sample, was placed on the agar layer, as shown in Stage 1 of Figure 1. Milk containing no added antibiotics was used as a

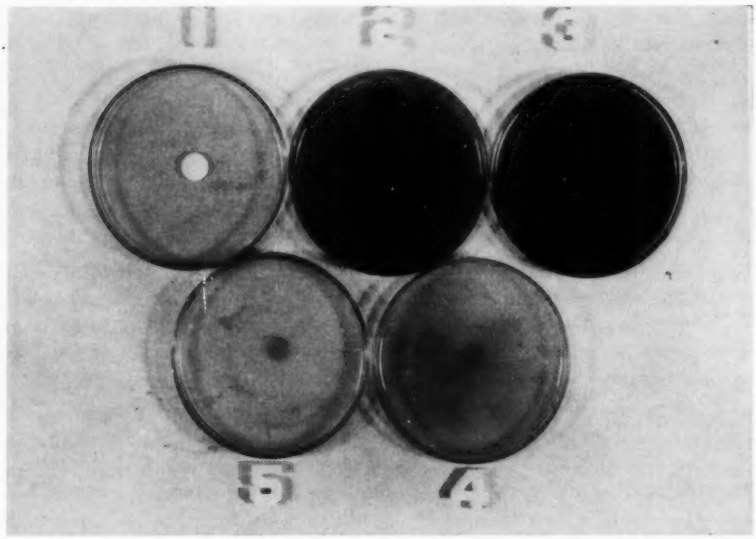

FIG. 1. Various stages involved in performing the rapid test for detecting antibiotic activity in milk.

control. The plate was then incubated at 40° C. for about 40 min., to permit the milk from the disk to diffuse into the agar and to allow the initiation of the growth of the organisms. After this incubation period, the plate was withdrawn from the incubator and, using a pair of flamed forceps, the disk was removed from the plate.

Approximately 0.5 ml. of 0.084% resazurin solution was then pipetted onto the plate. The plate was rotated gently by hand until the entire surface was covered with the dye solution. As may be seen (Figure 1, Stage 2), the pipetting of the dye solution produced irregular and patchy areas of color. However, the dye diffused readily into the agar and within 5–10 min. irregular patches of color

disappeared and a uniform violet surface was obtained (as shown in Stage 3). The entire surface was then covered with approximately 10 ml. of heavy liquid paraffin or mineral oil.

The plates were returned to the incubator at 40° C. and observed periodically. Within 10–90 min., the reduction of the dye was initiated by the organisms growing in the agar, except at the place where antibiotics from the milk had diffused in the agar (as shown in Stage 4, Figure 1). The mineral oil covering markedly speeds the reduction of the dye and also prevents reoxidation of dihydroresorufin to resourufin. Within 40–150 min., the dye was reduced further, most of the surface became light-red, and latent zones of inhibition became visible as violet or deep-red (as in Stage 5, Figure 1). Upon prolonged incubation, the inhibition zones were reduced to pink, while the pink plate background became light-pink or colorless. Eventually, the zones disappeared after 6–8 hr., due to the overgrowth of the organisms.

Several trials were conducted to obtain good color contrast within a short period of time. The factors affecting the time for completion of the test were the type, age, and concentration of the inoculum, amount of agar in each plate, and resazurin concentration. Although Figure 1 shows only one disk in each plate, it is possible to run from three to four samples in each plate.

RESULTS AND DISCUSSION

Fresh raw milk obtained from the University dairy was inoculated with appropriate quantities of freshly prepared antibiotic solution and tested for antibiotic activity by the procedure described. With each set of from three to four samples, a raw milk sample without any added antibiotic was run in duplicate or triplicate as a control. A total of 76 trials was conducted and the results are summarized (Table 1). The concentrations of penicillin reported represent international units[3] of penicillin per milliliter and other antibiotics represent γ or micrograms per milliliter of milk. For the sake of brevity, the concentrations of all of the antibiotics have been termed as parts per million.

It may be observed that when *S. aureus* was used as the test organism, 0.04–0.06 p.p.m. of penicillin, aureomycin, terramycin, or achromycin, and 0.17 p.p.m. of streptomycin could be detected in milk in 2–3 hr., including the 40-min. period required for the first incubation. When *L. bulgaricus* was used as the test organism, 0.04–0.06 p.p.m. of penicillin, aureomycin, terramycin, or achromycin, and 0.11 p.p.m. of streptomycin, could be detected in 1.6–1.9 hr., including the 40-min. period required for the first incubation. Both organisms were found to be suitable for detecting extremely small concentrations of antibiotics. In general, the colored inhibition zones formed with *S. aureus* were relatively clearer but took a slightly longer time to develop than with *L. bulgaricus*. Concentrations of antibiotics lower than those reported in Table 1 were also tested, but these lower concentrations could not be detected in milk.

[3] One international unit of penicillin weighs 0.6 microgram (*Penicillin Brochure with Annotated Bibliography*. Merck and Co., Inc., Rahway, New Jersey. 1945.)

TABLE 1

Levels of antibiotics detectable in raw milk with the rapid test, using Staphylococcus aureus *and* Lactobacillus bulgaricus *as test organisms*

Staphylococcus aureus					*Lactobacillus bulgaricus*				
Antibiotic	Range	Trials	Time for zone development		Antibiotic	Range	Trials	Time for zone development	
	(p.p.m.)	*(No.)*	*(Range hr.)*[a]	*(Av. hr.)*[a]		*(p.p.m.)*	*(No.)*	*(Range hr.)*[a]	*(Av. hr.)*[a]
Penicillin	0.04–1.67	10	1.4–3.1	2.3	Penicillin	0.04–1.67	16	1.5–2.5	1.9
Streptomycin	0.17–0.68	7	1.6–2.6	2.0	Streptomycin	0.11–0.50	10	1.4–2.3	1.9
Aureomycin	0.04–0.60	5	1.2–2.5	1.9	Aureomycin	0.06–0.50	5	1.4–2.5	1.8
Terramycin	0.05–0.50	4	2.1–3.2	2.9	Terramycin	0.05–0.30	6	1.3–1.8	1.6
Achromycin	0.06–0.50	6	2.0–3.1	2.5	Achromycin	0.06–0.50	7	1.5–2.5	1.8

[a] Includes 40 min. required for the first incubation.

In a few instances during these trials, colored zones of inhibition were observed with control milk samples containing no added antibiotics. Upon prolonged incubation, these zones disappeared. It was felt that such false zones could result from other inhibitory substances inherent in milk.

Natural inhibitors in milk. In order to investigate further whether there existed any natural inhibitors in milk received from untreated cows, and to determine whether this test would detect such inhibitors, 15 milk samples were collected from individual healthy cows. These cows either had never received any antibiotic treatment or had not received any during at least 4–6 mo. before collection of the samples. Each of the 15 milk samples was divided into two lots. One lot was held raw and the other was heated at 170° F. for 2 min. or at 180° F. for 1 min. Antibacterial activity was determined by the resazurin disk assay method. It was observed that one of the raw milk samples possessed antibacterial activity, but that when that sample was heated it had lost its inhibitory properties.

The fact that in a few cases color zones of inhibition were obtained with raw milk without added antibiotics reveals that this method when applied to raw milk can detect not only the antibiotics but also the natural inhibitors that may be present in milk. In general, the heating of the samples is not required in order to determine whether the milk possesses any starter inhibitory properties. However, when it is desired to establish whether the inhibition is due to antibiotics or some heat-labile natural inhibitors present in milk, the heating of the samples may facilitate the differentiation.

Detection of antibiotics in heated milk. Based upon the above observations, additional trials were conducted in which 25 samples of milk were heated to 170° F. for 2 min. or to 180° F. for 1 min. after the addition of antibiotics, quickly cooled to below 40° F., and examined for antibacterial activity by the rapid method. Heating of milk was carried out after the addition of the antibiotics, to simulate the conditions that might occur in the case of raw milk samples containing antibiotics and tested by this technique.

Table 2 presents the concentration levels of antibiotics that could be detected by this method in milk samples after they were heated. The lowest concentrations that could be detected in the heated milk were 0.1 p.p.m. of penicillin, 0.3 p.p.m. of streptomycin, 0.06 p.p.m. of aureomycin, or 0.28 p.p.m. of terramycin.

TABLE 2

Levels of antibiotics detected in heated milk with the rapid test, using Staphylococcus aureus *as the test organism*

Antibiotic	Concentration	Trials	Heat treatment	Time for zone development	
	(p.p.m.)	*(No.)*		*(Range hr.)*[a]	*(Av. hr.)*[a]
Penicillin	0.1 –1.2	7	170° F.—2 min.	2.2–4.2	2.8
Streptomycin	0.3 –0.5	6	180° F.—1 min.	2.3–3.6	2.5
Aureomycin	0.06–0.5	6	180° F.—1 min.	1.5–2.2	2.1
Terramycin	0.28–1.0	6	180° F.—1 min.	2.2–2.5	2.4

[a] Includes 40 min. required for the first incubation.

Table 3 presents the minimum concentrations of various added antibiotics that could be detected in raw and in heated milk. To detect antibiotics in the heated milk by this method, slightly higher concentrations of antibiotics had to be present in the milk before it was heated. This should be expected, since upon heating of milk the antibiotics are inactivated partially, as reported by Shahani *et al. (11)*. Nevertheless, the test is of value for determining whether or not the milk could be used for the manufacture of cultured products. The test may be applied to raw milk when the milk is intended for the manufacture of raw milk cultured products, and to milk after heating if it is intended for use in the manufacture of cultured products from heated or processed milk.

TABLE 3

Minimum concentrations of antibiotics detected in raw and heated milk with the rapid test, using Staphylococcus aureus *as the test organism*

Antibiotic	In raw milk [a]	In heated milk [b]
	(p.p.m.)	*(p.p.m.)*
Penicillin	0.04	0.1
Streptomycin	0.17	0.3
Aureomycin	0.04	0.06
Terramycin	0.05	0.28
Achromycin	0.06	.....

[a] From Table 1.
[b] From Table 2 (antibiotic added prior to heating the milk).

The presence of a high count of penicillin-resistant, gram-negative organisms, including *Escherichia coli*, in raw milk containing penicillin could reduce resazurin and mask the inhibitory effect of penicillin. However, occurrence of such a high gram-negative count necessary to reduce resazurin in such a short time would be extremely rare, even in the case of cheese milk. As will be recalled, the test is based upon the inhibition of the reduction of resazurin by a large bacterial population inoculated in the agar and not by the organisms present in milk.

Also, a question may arise as to how, in raw milk testing, it would be possible to distinguish the inhibitory effect produced by a bacteriophage from that produced by antibiotics. It is felt that under normal conditions the number of bacteriophage particles in the minute quantity of milk (approximately 0.05 ml.) on paper disk would be extremely small, and would not affect the growth of the test organisms in the short time required for completing the test.

Detection of sanitizing agents in milk. Another source of starter failure could be the presence of high concentrations of sanitizing agents in milk. Fresh raw milk was inoculated with various levels up to 3 p.p.m. of a quaternary ammonium compound (alkyl dimethyl benzyl ammonium chloride) or 25 p.p.m. of a chlorine compound (calcium hypochlorite) and tested. The concentrations of the sanitizers represent the concentrations of the active sanitizing ingredients only. These concentrations of sanitizing agents did not produce zones of inhibition, indicating that normal residual concentrations of sanitizers present in milk would not be detected by this test. Neal and Calbert *(6)*, using 2,3,5–triphenyltetrazolium chloride as an indicator, observed only slight inhibition of a

starter culture in milk containing 10 p.p.m. of a quaternary ammonium compound or 70 p.p.m. of hypochlorite.

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A COMPARISON OF BACTERIAL GROWTH IN MILK AND IN MILK-WATER COMBINATIONS

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SUMMARY

Bacterial development in milk and in four milk-water combinations was studied by inoculating autoclaved samples with raw milk or pure cultures and making plate counts after incubation at 32° C. In the raw milk inoculum experiments, the counts recorded after 6 hr. of incubation were variable. However, there was a trend toward higher counts in certain milk-water combinations than in undiluted milk.

With six pure cultures, after 6 hr. of incubation, counts tended to be higher in certain milk-water combinations than in undiluted milk, except for one culture. The increases always followed some type of a curvilinear trend and were statistically significant compared to zero dilution with two cultures, of near significance with a third, and nonsignificant with two others. With the remaining culture *(Streptococcus lactis)*, counts decreased significantly with each succeeding increment of water dilution. After 24 hr. of incubation, none of the counts from milk-water combinations was significantly higher than in undiluted milk. In all cases after 6 hr. of incubation, and in all but one after 24 hr. of incubation, counts on samples containing 98% water were markedly lower than on undiluted milk. Presumably, the addition of certain amounts of water to milk created more suitable conditions for initial development of some bacterial species than was provided in undiluted milk.

Development of microorganisms is well recognized as being influenced by physical and physico-chemical factors. However, Hendlin *(2)* states that little attention has been given to the significance of physical environment in the nutrition of microorganisms and that the importance of this aspect is becoming increasingly evident.

Olson *et al. (4)* found that bacterial development was more rapid in recombined milk than in the concentrated milk from which it was made. The increase in bacterial growth was inversely proportional to the concentration of milk solids. Simonart *et al. (5)* reported that, in normal fluid milk, *Streptococcus lactis* rapidly inhibited coliform organisms, but that in milk greatly diluted with water there was no inhibition. According to Keilling and Camus *(3)*, aroma-producing bacteria from butter developed more readily in diluted milk and in diluted buttermilk than in these products when undiluted. In the foregoing instances, it is probable that such factors as changes in osmotic pressure, colloidal characteristics, emulsion structure, and concentration of nutrients were involved.

During warm weather, the rapid putrefaction that often occurs in milk-water residues when left in utensils suggests more active bacterial development than takes place in milk. Although such cases may result largely from the particular bacterial types involved, growth stimulation through dilution with water also

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might be a factor. Since only limited information could be found on the effect of water dilution of milk on bacterial growth, and since some physical or physico-chemical influences might be expected, a study was made to compare bacterial development in milk and in various milk-water combinations.

METHODS

Experiments with raw milk as inoculum. Three trials were made with triplicate sets of samples, involving nine determinations for each milk-water combination. In each trial, five combinations of fresh whole milk and water were prepared to contain: 0, 25, 50, 75, and 98% added distilled water, respectively. Each different milk-water combination was dispensed in triplicate 100-ml. samples (Sets A, B, and C) in 6-oz. prescription bottles and autoclaved for 20 min. at 15 lb. of pressure. After cooling, all samples were stored overnight in a refrigerator.

Samples were inoculated after being tempered to 32° C. Inoculum consisted of raw whole milk that had been incubated 3-4 hr. at room temperature, with a different lot being used in each trial. The five different samples, in triplicate, were inoculated at the rate of 0.1%, then incubated at 32° C. This temperature was selected as representative of warm weather and also because it permitted definite bacterial development in a relatively short time. After 6 hr. of incubation, bacterial counts were made in duplicate on each sample by standard plate methods, except that the medium recommended by Donovan and Vincent *(1)*, for determining total numbers and for detecting acid-producing, alkali-producing, and proteolytic bacteria in milk, was used. The three sets of samples (A, B, and C) were plated consecutively, with 25-35 min. being required to complete the procedure.

Experiments with pure cultures as inoculum. To determine whether different types of bacteria responded differently, three trials were made with each of six pure cultures isolated from milk. Trials were conducted in duplicate, providing six determinations for each milk-water combination with each culture.

The general procedures were the same as in the experiments with raw milk inoculum, with the following exceptions: The milk-water combinations were prepared in duplicate instead of in triplicate, since the latter were shown statistically to be unnecessary. Fresh skimmilk was used to replace whole milk, since some fat clumping occurred with the latter when shaken for plating. The pH values of the samples dropped somewhat during autoclaving, and were adjusted back to pH 6.6–6.7 with sterile normal NaOH. The pH value of Number 5 dilution (98% water) usually was near 7.0, and was not adjusted, as such pH was not considered inhibitory to growth of the bacteria employed as inocula.

Cultures used as inocula were isolated from raw milk less than 1 wk. prior to testing, and were subcultured in litmus milk. In the isolations, colonies were picked from agar plates (Donovan and Vincent medium) more or less at random, but seemingly rare or unusual types were avoided. Inoculation of samples was at the rate of 1% of a 1:10,000 water dilution of a 24-hr. milk culture grown at 32° C. Platings of the samples were made after both 6 and 24 hr. of incubation.

Description of cultures used. The cultures isolated from milk and used in the experiments were subsequently studied and tentatively identified as types described in Bergey's *Manual* (1948) as follows:

Culture A — *Flavobacterium brevi*
Culture B — *Flavobacterium brevi*
Culture C — *Streptococcus lactis*
Culture D — *Paracolobactrum intermedium*
Culture E — *Klebsiella pneumonia* (reaction in litmus milk resembled coliform organisms)
Culture F — *Bacterium minutaferula*

Statistical treatment of data. The statistical part of this study was designed to answer three questions: *(a)* At what point, or points, along the dilution scale of measurement has the mean bacterial count increased (or decreased) significantly relative to the mean count at zero dilution? *(b)* If the mean bacterial count does increase significantly with some dilution (or dilutions), at what increased dilution does it drop significantly—as it obviously must for *some* dilution? *(c)* Is there a rather constant and mathematically describable type of trend in mean bacterial count as the dilution increases from zero to near 100% and, if so, what is this type of trend?

The nonnormality of the frequency distributions of bacterial counts made it necessary to transform the counts to log counts. Thereafter, preliminary graphic analysis indicated that question *(c)* should be answered negatively. However, this conclusion was checked by an orthogonal polynomial analysis. Although a significant quadratic component was found in the trend of log count with increased dilution from 13 of the 15 situations, the quadratic trend was variously oriented with respect to the dilution axis, so that no useful general type of trend could be described. Sometimes, there was no maximum on the curve; a few other times there was a suitable maximum and, in other cases, the apparent maximum was to the left of the obvious peak of the trend of log count with increasing dilution. This last situation occurred whenever a gradual increase in count was followed by an abrupt drop in count for a higher dilution—which frequently occurred between dilutions 4 and 5.

Consequently, it was decided that t-tests between the mean log count at zero dilution and succeeding dilutions, and between the peak (if any) mean log count and higher dilutions, would best serve the purposes of this study.

RESULTS

Experiments with raw milk as inoculum. The effect of water dilution on bacterial growth as measured by logarithms of bacterial counts made after 6 hr. of incubation is indicated (Figure 1). In general, a higher count was obtained in those samples containing 25% and/or 50% of added water than in undiluted milk. At higher dilutions, the counts generally decreased as dilution increased, with a marked decrease at the 98% dilution.

In analyzing the data, it was noted that the general level of counts and the sampling variance increased progressively from the Set A samples to the Set C

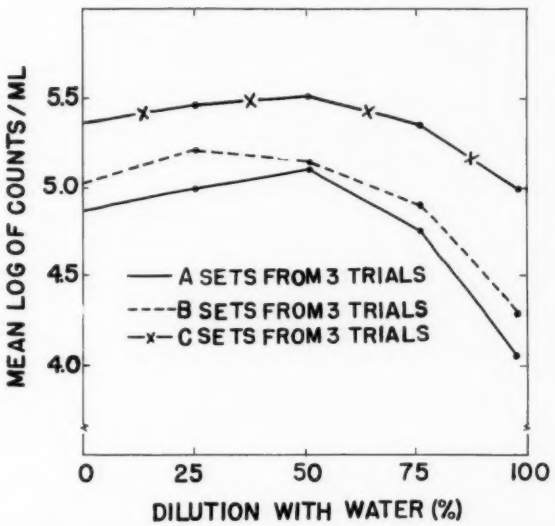

FIG. 1. Mean logs of bacterial counts on milk and on milk-water combinations after 6 hr. of incubation with raw milk inoculum.

TABLE 1

Summary of log means of bacterial counts on different milk-water combinations[a]

(Raw milk inoculum; 6-hr. incubation)

Sets of samples	Dilution:[b] 1		2		3		4		5
A	4.86		4.97		5.09	ns	4.74	*	4.06
			ns						
B	5.02	ns	5.20		5.14		4.90	*	4.27
					ns				
C	5.34	*	5.45	ns	5.49	*	5.34	*	5.00

ns = Statistically nonsignificant.
* = Statistically significant at or beyond the .05 level.
[a] = Values are means of three trials.
[b] = Dilution: *1*) no added water, *2*) 25% water, *3*) 50% water, *4*) 75% water, *5*) 98% water.

samples in each trial. However, the sample-within-set variance was homogeneous from set to set. Accordingly, the data from each set of samples were analyzed separately. Results from the A sets in the three trials were then grouped together, as were also results from the B sets and the C sets. The mean log count for each dilution within each set of samples is given (Table 1). Statistically significant changes in mean log count with increasing dilution are indicated. Although there was an apparent increase in count at certain dilutions in each case, the increases within the A sets and the B sets were not statistically significant. In the C set, however, there was a significant increase in count with 25% dilution, little additional increase at 50% dilution, then successive significant decreases at 75 and

98% dilutions ($P \leq .05$). An orthogonal polynomials analysis of the data in Table 1 confirms the curvilinearity of the trend of log count with increased dilution, but it does not produce a general type suitable to all three sets of data.

When counts were higher at certain dilutions than in undiluted milk, relatively high proportions of alkali-producing organisms and/or proteolytic organisms were present. On the other hand, when acid-producing types were more common, there was little or no tendency for counts to be higher in diluted samples. Variations in growth resulting from dilution of milk may have arisen partly from the types of bacteria in the raw milk used as inoculum, with different types exhibiting different responses to dilution.

Experiments with pure cultures as inoculum. The response of pure cultures to water dilution of milk, as shown by bacterial counts made after 6 hr. of incubation, varied with different cultures. Mean log counts for each culture at the various dilutions (three trials in duplicate) are presented in Table 2, and are plotted in Figure 2. There was a general trend to more rapid growth in milk containing some amount of added water than in milk alone, except with Culture

TABLE 2

Summary of log means of bacterial counts on different milk-water combinations[a]

(Pure culture inoculum; 6-hr. incubation)

Culture	Dilution:[b] 1		2		3		4		5
A	3.96		4.11	*	4.28	ns	4.33	*	3.17
B	3.87	*	3.98		3.89	*	3.81	*	3.14
C	6.58		6.49	*	6.42	*	6.16	*	5.83
D	5.53		5.90		5.97		6.01	*	5.24
				ns					
E	5.85	near *	6.01		5.97	*	5.82	*	5.15
F	4.41		4.66	ns	4.77	ns	4.70	*	3.64

ns = Statistically nonsignificant.
* = Statistically significant at or beyond the .05 level.
[a] = Values are means of six determinations.
[b] = Dilution: *1)* no added water, *2)* 25% water, *3)* 50% water, *4)* 75% water, *5)* 98% water.

C. However, the dilution at which highest counts were obtained differed among cultures. Of the six cultures, the increase in count was statistically significant for Cultures A and B ($P \leq .05$), and was near significance for Culture E ($P = .06$). With Culture A, the highest count was obtained from the 75% water dilution; and with Cultures B and E, from the 25% dilutions. Although Cultures D and F showed trends to higher counts at some dilution, with highest counts recorded at the 75 and 50% dilutions, respectively, these increases were not statistically significant. Culture C (*S. lactis*) was the only culture with which the count was highest in undiluted milk and continually decreased with increased dilution. All cultures dropped off markedly in count at the 98% dilution. As some additional indication of a trend to more rapid growth in diluted milk, odor

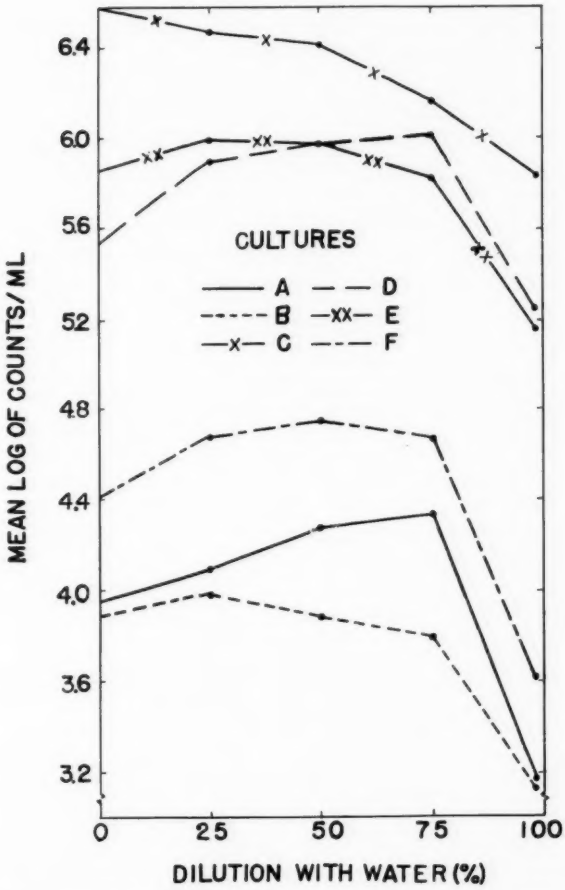

FIG. 2. Mean logs of bacterial counts on milk and on milk-water combinations after 6 hr. of incubation with pure cultures (means from three trials in duplicate for each culture).

defects usually developed at certain dilutions sooner than in undiluted milk. Again, the orthogonal polynomials analyses confirmed the curvilinearity of the trend of log count with dilution, but did not produce a general type of trend suitable for all cultures.

After 24 hr. of incubation, the apparent stimulatory effect of water dilution on growth of some cultures had generally disappeared (Table 3 and Figure 3), but the log count-dilution trends still were curvilinear except with Culture F. Although, in some cases, counts were slightly higher at certain dilutions than in undiluted milk, the differences were not statistically significant. With all cultures except F, there was a significant drop in count in the 98% dilutions.

TABLE 3

Summary of log means of bacterial counts on different milk-water combinations[a]

(Pure culture inoculum; 24-hr. incubation)

Culture	Dilution:[b] 1		2		3		4		5
A	8.12	ns	8.27	ns	8.26	ns	8.22	*	7.99
B	8.17	ns	8.23	ns	8.15	ns	8.02	*	7.73
C	9.37		9.26		9.05	*	8.62	*	7.70
D	8.91	ns	8.96	ns	8.88	ns	8.68	*	8.20
E	9.05	ns	8.97		8.97	*	8.59	*	7.74
F	7.73		7.72	near	7.72		7.61	ns	7.62

ns = Statistically nonsignificant.
* = Statistically significant at or beyond the .05 level.
[a] = Values are means of six determinations.
[b] = Dilution: *1*) no added water, *2*) 25% water, *3*) 50% water, *4*) 75% water, *5*) 98% water.

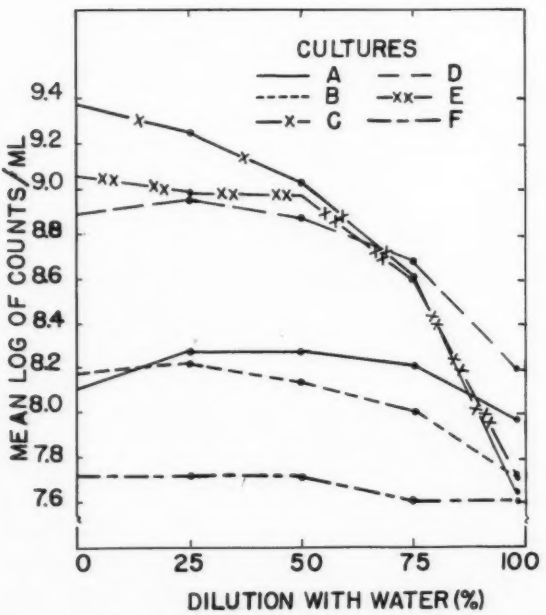

FIG. 3. Mean logs of bacterial counts on milk and on milk-water combinations after 24 hr. of incubation with pure cultures (means from three trials in duplicate for each culture).

DISCUSSION

In the experiments with raw milk inoculum, a partial explanation that may be offered for the increase in count levels from the A sets to the C sets is the time elapsed in the plating procedure. Presumably, the organisms were in an active growth stage and increased in numbers during the period that elapsed in plating the three sets of samples. Although the same amount of inoculum was added to all samples in each trial, the numbers of organisms added would vary some between samples. However, this may be considered a random variation.

The variations in growth response to water dilution when raw milk was used as inoculum are probably partly explained by the results subsequently obtained with pure cultures, where responses varied with species. It is of interest that *S. lactis* (Culture C) was the only organism among the six tested that developed more slowly with succeeding increments of water dilution. This reaction might not be unexpected from an organism adapted to milk as a habitat and probably having different nutritional requirements than the others. Although all species tested were isolated from milk, some are not considered to be among the more common types in milk. However, it is emphasized that in the isolations, seemingly rare or unusual colony types were avoided and that the identity of the organisms was not determined until the end of the experiments. While organisms that are not fastidious in nutritional requirements might be expected to grow well in diluted milk, this characteristic would not explain the stimulated growth frequently obtained by dilution. It may be that these forms were less suitably adapted to a milk environment than others; hence, grew more rapidly in some milk-water combination than in undiluted milk. Nevertheless, the results obtained with raw milk inoculum suggest that bacteria with such characteristics may not be uncommon in milk.

Various reasons may be suggested for the apparent stimulatory effect of dilution on growth of certain bacteria. It is possible that some substances in the milk, inhibitory to the organisms involved, were diluted out. However, the autoclaving of the milk, which is known to inactivate some inhibitory substances, somewhat limits this possibility. Dilution would reduce the concentration of nutrients. Possibly, there is an optimum concentration for most rapid development. Modifications in such physico-chemical factors as osmotic pressure and colloidal characteristics might affect the nutrition of the bacterial cells.

The more rapid bacterial growth during storage [reported by Olson *et al. (4)*] in recombined milk than in the original concentrate, appears to be analogous to the results herein reported, although at higher levels of milk solids concentration. It is possible that some further dilution of the recombined milk would have produced an additional increase in bacterial counts.

To provide control of numbers and/or types of organisms initially present, the experiments were conducted with autoclaved milk. It is possible that the relationships found would differ in raw milk or in moderately heated milk. Nevertheless, the results seem to permit certain speculations. The tendency for some bacterial species to develop more rapidly in milk-water combinations than in undiluted milk may be of significance in dairy sanitation. This characteristic

would be expected to accelerate decomposition of milk-water residues and contribute to the prevalence of off-odors. Prolonged presence of milk-rinsings on milk-room floors or in equipment might have a selective influence on the prevalent bacterial flora. Where there are delays between initial rinsing of equipment and final cleaning, the efficacy of sanitizing practices might be affected.

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BOVINE FETAL HEMOGLOBIN. I. POSTNATAL PERSISTENCE AND RELATION TO ADULT HEMOGLOBINS[1]

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SUMMARY

The postnatal persistence of bovine fetal Hemoglobin (F) and its relation to the adult Hemoglobins A and B were investigated by electrophoretic methods. At birth, F made up 41 to 100% of the total hemoglobin of 23 calves. The amount of F diminished rapidly and was replaced entirely by Hemoglobin A in 14 bull calves and four heifer calves at the average ages of 65 and 97 days, respectively. The disappearance of Hemoglobin F from the blood of five calves (Guernseys, Jerseys, Brown Swiss) was obscured by the simultaneous appearance of Hemoglobin B, which has the same electrophoretic mobility as F under the conditions used. Comparisons of electrophoretic mobilities in the pH range 6.5–9.0 showed that Hemoglobin A has a greater net positive charge than B and F throughout, but that the net positive charge of Hemoglobin B is appreciably greater than that of F only below pH 7.4. Above this pH, B and F migrate on paper electrophoretic strips at essentially the same rate.

It has been known for a long time that the hemoglobins of the fetus and adult of various species differ. Körber *(19)*, in 1866, first demonstrated differences in the resistance of fetal and maternal hemoglobins to denaturation by acids and alkalis. More recently, fetal and adult hemoglobins have been found to differ in such properties as solubility *(15, 17, 24)*, electrophoretic mobility *(1, 4, 13, 25)*, ultraviolet absorption spectrum *(4, 16)*, crystal form *(15, 18)*, oxygen dissociation curves *(3, 20, 21)*, behavior of monomolecular layers *(14)*, and amino acid composition *(7, 10, 23)*. Some of the comparisons were made with human hemoglobins, others with sheep, goat, and cattle pigments.

Although observations to determine the postnatal duration of fetal hemoglobin in the blood of human infants have been reported *(4, 14)*, the authors are not aware of any studies with this specific objective having been made with other species. The object of the work reported here was to determine how long the fetal hemoglobin of the calf persists postnatally, and to determine its electrophoretic properties in relation to bovine Hemoglobin A, the more common permanent type, and Hemoglobin B, a second adult type recently found to occur either alone or simultaneously with A in some individuals of certain breeds of cattle (Bangham, *2;* Salisbury and Shreffler, *22;* Grimes, Duncan, and Lassiter, *8*).

Our earlier tentative designation of F *(8)* for bovine Hemoglobin B was based on the possibility that it was identical to fetal hemoglobin. The findings presented in this report point out the need for a revision of our nomenclature.

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EXPERIMENTAL PROCEDURE

Twenty-three calves were used for the determination of postnatal persistence of fetal hemoglobin (F). Heparinized or oxalated blood samples were obtained on the day of birth or as soon thereafter as possible, and at biweekly or shorter intervals until fetal hemoglobin was absent. Four per cent hemoglobin solutions were prepared by a modification of the method of Drabkin *(6)*. The hemoglobin preparations were usually used on the day of collection, but if not, were stored frozen for not more than two days before use. Electrophoretic separation was carried out on a ridge-pole type (Spinco) paper electrophoresis apparatus with a barbital buffer of pH 9.0 and ionic strength of 0.1. Sample volume was 3–5 μl. applied in a 2.5-cm. streak across the paper strip. A constant direct current was applied to the eight Whatman 3MM paper strips for 16 hr., while the cell was kept in a refrigerator at 4° C. The strips were dried, dyed with bromophenol blue, washed with dilute acetic acid and acetate buffer by the procedure recommended by Block, Durrum, and Zweig *(5)*. The strips were quantitatively evaluated by means of an automatic recording and integrating densitometer. Analyses were always made in duplicate and the results averaged.

The same procedure was followed for comparing the electrophoretic behavior of fetal hemoglobin with Hemoglobins A and B, except that the more stable carboxyhemoglobins were used in place of oxyhemoglobins. The oxyhemoglobin solutions were prepared as before from the blood of a Holstein cow (A), a Jersey cow (B), and a four-day-old Holstein calf. Carboxyhemoglobins were prepared by first deoxygenating the samples for 4 hr. in a vacuum desiccator, then filling the evacuated desiccator with carbon monoxide to atmospheric pressure and equilibrating for 4 hr. The hemoglobin solutions were stirred gently and continuously during the entire process by a magnetic stirrer. Separate small aliquots were stored frozen and discarded after thawing and use. Phosphate buffers of 0.1 ionic strength were used for pH 6.5, 7.0, 7.4, and 8.1. For pH 8.6 and 9.0, 0.1 ionic strength barbital buffers were used. Duplicate samples of all three hemoglobin types were run simultaneously at each pH on two different occasions.

RESULTS AND DISCUSSION

Fetal hemoglobin was found to migrate in a reasonably narrow, well-defined band at a faster rate than Hemoglobin A at pH 9.0. Resolution of these two types was enhanced by the use of the relatively high ionic strength and pH, and especially by the low temperature. The electrophoretic patterns and density tracings reproduced in Figure 1 are typical of the separations obtained.

The amount of Hemoglobin F present at birth varied widely with individual animals, ranging from 41 to 100% of the total. The average was 87%. Synthesis of Hemoglobin A obviously had started in most cases before parturition.

In preparations from 18 calves, F diminished at a fairly regular rate and finally was replaced entirely by A. With 14 bull calves, including ten Holsteins, one Guernsey, one Brown Swiss, and two Ayrshires, this occurred at an average age of 65 days (range, 55–73 days). With four heifer calves, all Holsteins, the times required were 54, 105, 106, and 124 days, suggesting that sex may have

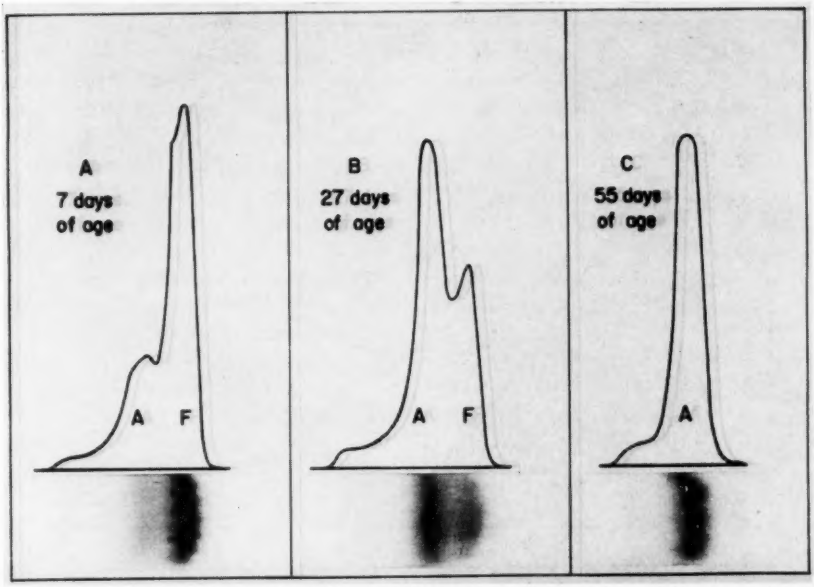

FIG. 1. Paper electrophoretic patterns and density tracings of hemoglobin from the same calf at 7, 27, and 55 days of age. Fetal hemoglobin (F) is the leading component. Migration was toward the anode.

some influence on the postnatal persistence of fetal hemoglobin. Curves 1 and 2, in Figure 2, illustrate the apparent differences. The observations of Bangham *(2)* and Salisbury and Shreffler *(22)* that fetal hemoglobin can be detected to about 3 mo. of age thus have been substantially confirmed.

Figure 2 also shows the results obtained with a Guernsey bull calf (Curve 3), in which the ratio between the two hemoglobin components approached 1.0 at 65 days and remained at this level. The same results were observed with two other calves, a Guernsey and a Brown Swiss. Fetal hemoglobin also apparently failed to disappear from the blood of two additional calves (a Guernsey and a Jersey), but unlike the cases just described, no Hemoglobin A was ever detected from the date of birth to 67 and 83 days of age when the observations were discontinued (Curve 4, Figure 2).

We reported previously *(8)* that Hemoglobin B had been found alone or in combination with A only in some Jerseys and Guernseys, and that in animals having both types the ratio of A to B was invariably close to 1.0. Salisbury and Shreffler *(22)* also found two hemoglobins in some Brown Swiss cattle. Paper electrophoretic patterns in pH 9.0 barbital buffer of hemoglobin preparations from calves in which fetal hemoglobin has diminished to about half of the total, and from cows possessing both A and B, are completely similar. Strips with fetal hemoglobin only and Hemoglobin B only are also indistinguishable. The data on migration distances of the three hemoglobins in pH 9.0 barbital buffer (sum-

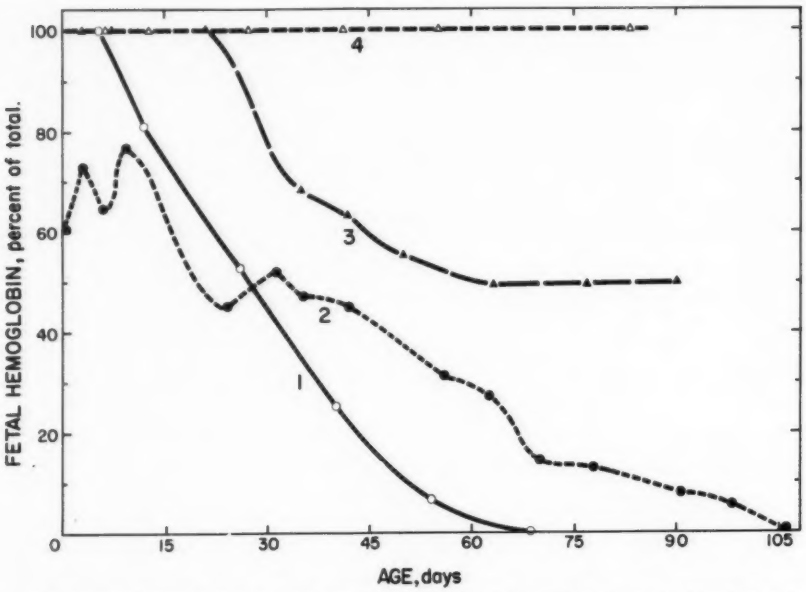

FIG. 2. Variation in the rate of disappearance of fetal hemoglobin in the blood of calves. Curve 1, Holstein bull; Curve 2, Holstein heifer; Curves 3 and 4, Guernsey bulls.

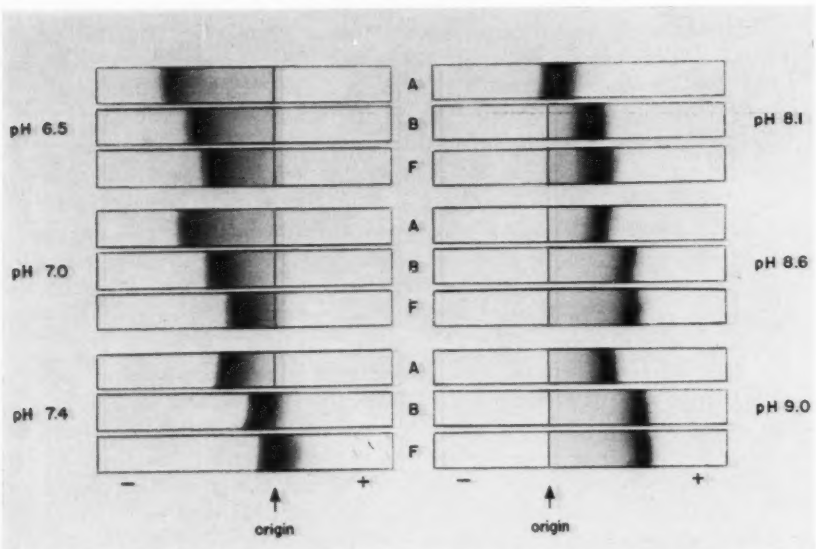

FIG. 3. Comparison of electrophoretic migration of bovine Hemoglobins A, B, and F at several pH levels.

marized in Table 1) show that the mobilities of B and F are statistically identical.

The similar electrophoretic properties of Hemoglobins B and F and the results with the five Guernsey, Jersey, and Brown Swiss calves in which fetal hemoglobin seemingly failed to disappear suggested the possibility that these proteins might be identical. To test this possibility, the electrophoretic mobilities of Carboxyhemoglobins A, B, and F were compared at pH 6.5, 7.0, 7.4, 8.1, 8.6, and 9.0. The results (shown by the typical strips in Figure 3) indicate that B and F are not the same, although their mobilities are practically identical on the alkaline side of their isoelectric points. Definite differences appear only below the isoelectric points. Both B and F have mobilities appreciably different from A throughout the pH range used. These mobility relationships are illustrated (in Figure 4), in which migration distances are plotted against pH.

TABLE 1

Paper electrophoretic migration distances of fetal and adult bovine hemoglobins

Hemoglobin type	Number of observations	Migration distance	
		Mean	S. D.
		(cm.)	
A, calf	139	2.4	± 0.28
A, adult [a]	66	2.4	± 0.16
B [a]	30	3.9 [c]	± 0.14
F	118 [b]	3.8 [c]	± 0.31

[a] Values taken from previous report *(8)*.

[b] Values from samples containing less than 10% fetal hemoglobin were omitted since, in these cases, resolution was not as good.

[c] The difference between these values is not statistically significant ($P = 0.1$).

The disappearance of Hemoglobin F in the five calves mentioned above thus was obscured by the simultaneous appearance of Hemoglobin B at the same place on the electrophoretic patterns.

Although the curves in Figure 4 show clearly the mobility ratios between the hemoglobins, it should be pointed out that the data plotted are not the true values of migration distances, because electro-osmotic flow was not measured. Since electro-osmotic flow is from anode to cathode, the curves would be shifted downward if it were taken into account. However, the same mobility ratios would hold at each pH value, because electro-osmotic flow is dependent not on the type of sample but on factors which are constant from strip to strip at any one pH.

The structural differences which bring about the differences in electrophoretic behavior of the three hemoglobins can not be deduced from the data presented. Hemoglobin A has a greater net positive charge than B and F throughout the pH range studied, and Hemoglobin B has a greater net positive charge than F only below the isoelectric point. Above this pH, B and F apparently have the same charge. Ingram *(12)* and Hunt and Ingram *(11)* have recently presented strong evidence that the electrophoretically distinct human Hemoglobins A, S, and C differ by only a single amino acid residue out of nearly 300 in the half-molecule. A glutamic acid residue in Hemoglobin A is replaced by valine in

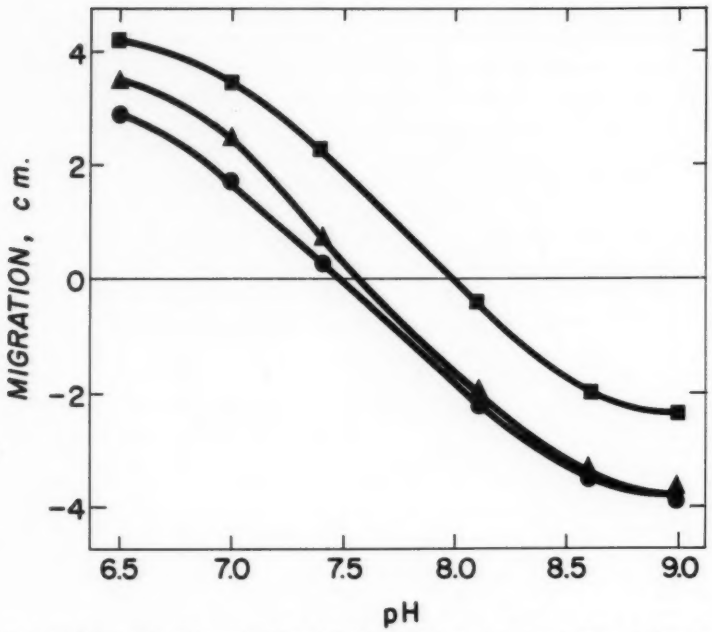

FIG. 4. Migration distances of Carboxyhemoglobins A, B, and F as a function of pH. At points above the zero line, migration is positive, i.e., toward the cathode. The observed migration distances were not corrected for electro-osmotic flow.

Hemoglobin S, and by lysine in Hemoglobin C. These very small changes apparently are sufficient to account for differences in electrophoretic mobility of the same order of magnitude as that found between bovine Hemoglobins A and B. The structural differences in the bovine hemoglobins conceivably could be equally small.

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INTERRELATIONSHIPS OF MILK TEMPERATURE, DILUTION, AND CURD FORMATION IN THE RESPONSE OF CALVES TO WHOLE MILK DIETS [1]

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SUMMARY

Twenty-four calves (16 Holstein and eight Jersey) were employed in a 2^3 factorial experiment to evaluate the influence of milk temperature (100 or 50° F.), dilution (none or containing 25% water), and curd formation (normal or prevented with sodium citrate) as factors in the feeding of calves. Calves 14 to 42 days of age were fed Jersey milk exclusively at 10 to 14% of initial body weight daily. Diarrhea was no problem and health and condition of calves were generally good. Body weight gains and efficiency of feed utilization were not significantly different for the two temperatures, dilution levels, or curding potentials. Significant interactions were found between dilution and temperature and between dilution and curd formation. The difference in gains between milk fed at the two temperatures was significantly greater ($P < 1\%$) when fed diluted than when fed undiluted. (Of these combinations diluted, warm milk gave poorest results.) Feed efficiency was significantly affected in the same manner ($P < 5\%$). The difference in gains between calves fed citrated and noncitrated milk was significantly greater ($P < 1\%$) when the milk was diluted than when undiluted. (Of these combinations, poorest gains were on diluted, curd-forming milk.) Feed efficiency was significantly affected in the same manner ($P < 1\%$).

Many of the practices currently recommended and used in the feeding of milk diets to calves have not been subjected to thorough evaluation. It is generally recommended that milk should be near body temperature when fed to calves and that it should be fed with regularity and little variation in temperature. Walker *(15)*, however, reported that satisfactory weight gains were made by three calves fed milk at 0° C. from birth to 3 wk. of age. Dilution of milk with water has been suggested by Sheehy *(14)* as a means of avoiding or curing diarrhea. Gaunya *et al. (6)* noted that calves fed diluted colostrum gained less than those fed undiluted colostrum, but the difference was not significant. Others *(10)* reported that dilution had no effect on weight gains of young calves but appeared to reduce the incidence of scours. Though there is evidence *(2, 7)* suggesting that the formation of a curd is necessary to avoid digestive disturbances and scours, Owen and others *(12)* found that the milk clot per se did not have a significant effect on the consistency of the feces of young calves. Many milk replacers which do not form a curd in the stomach have been successfully employed in calf-feeding *(13)*. It appeared that a more complete evaluation of the influence of some of the physical properties of the milk diet on the health and performance of calves might have practical as well as fundamental significance.

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This study was designed to ascertain the independent and interacting effects of milk temperature (100 and 50° F.), dilution (0 and 25% added water), and curd formation (normal and prevented with sodium citrate) on the health, growth, feed efficiency, feces characteristics, and subsequent starter consumption of young dairy calves.

EXPERIMENTAL PROCEDURE

Animals. Twenty-four calves from the University of Arkansas dairy herd at Fayetteville were used in this study, which began in January, 1956. The design of experiment employed was a 2^3 factorial composed of blocks of four units, with the three-way interaction confounded *(3)*. Two replications of the design were filled with Holsteins and one with Jerseys. Females were assigned to one of the two blocks within each replicate, and males to the other. Blocks 1, 3, and 5 were composed of females and Blocks 2, 4, and 6, of males. Random assignment of calves to position within blocks was made according to birth dates. (Unhealthy calves, or those weighing outside the range, for Jerseys of 44–71 lb. and for Holsteins, 69–116 lb., were excluded.) Replicates I, II, and III were filled in that chronological order.

Diets. Calves received colostrum from their dams until four days of age, when moved to individual straw-bedded pens. Feeding of experimental diets was begun at 14 days of age, prior to which mixed milk from the Jersey herd (which averaged 5.4% fat during the period of this study) was fed twice daily (A.M. and P.M.), at the rate of about 8% of body weight. Approximately 70 mg. of terramycin was provided daily in the milk in the form of a terramycin supplement.[3] The basal dietary regime employed in the experiment was the same antibiotic-supplemented milk as just described. It was fed at the rate of 10, 12, and 14% of the 14-day body weight, for the periods of 14 to 21, 21 to 28, and 28 to 42 days of age, respectively, when the experiment was terminated. Eight diets were required in the testing of the three factors, curd formation (C), dilution (D), temperature (T), and their interactions.

DIET 1—Basal, warm (97–100° F.)
DIET 2—Basal, warm, and citrated (4 ml. of a 50% sodium citrate solution per pound of milk)
DIET 3—Basal, warm, and diluted (to contain 25% tap water)
DIET 4—Basal, warm, diluted, and citrated
DIET 5—Basal, cooled (47–52° F.)
DIET 6—Basal, cooled, and citrated
DIET 7—Basal, cooled, and diluted
DIET 8—Basal, cooled, diluted, and citrated

Sodium citrate was used, since it has been found to be effective in preventing curd formation in the abomasum of the calf *(12)*. According to in vitro dilution tests, the rate of dilution used in this study did not prevent coagulation but

[3] Bi-Con TM-10, guaranteed to contain 10 g. terramycin per pound, was supplied by Chas. Pfizer and Co., Inc., Brooklyn, New York.

did result in a soft curd. A curd was considered necessary with the diluted diet in order not to confound the effect of citrate.

Observations. Records were kept of milk temperature and consumption at each feeding. Body weights were taken initially when the calves were placed on experiment and weekly thereafter. Health and general appearance were noted daily and feces observations were made each A.M. and P.M. From 15 through 28 days of age samples of feces, for pH and moisture determinations, were collected daily (if obtainable) by manual stimulation of defecation.

Previous work has indicated increased intestinal acidity as the cause of diarrhea *(5, 8)*. Feces samples were collected in small tin containers and within approximately 30 min. pH measurements were made by mixing about 5 g. of feces with 20 ml. of distilled water and reading on a Beckman Model H-2 glass-electrode pH meter. The remaining portion of the sample was stored in the tin containers at approximately −20° C. until moisture determinations were made. Moisture content was measured as the loss in weight of samples held at near 105° C. in an electric air oven until constant weight was attained.

RESULTS AND DISCUSSION

Growth and feed efficiency. Weight gains of individual calves in each dietary group are shown (Table 1). Average daily gains ranged from 0.54 lb. for the group receiving warm milk which was diluted, to 1.43 lb. for the cold milk diet which was both diluted and had citrate added for curd prevention. Thus, all diets supported satisfactory growth.

Milk temperature, dilution, and curding as independent factors did not have a significant effect on weight gains or efficiency of milk utilization during the 14- to 42-day age period (Table 2). Others *(4, 15)* have also reported successful use of cold milk diets. Gaunya *et al. (6)* reported that calves fed undiluted colostrum made 25% more gain in weight than calves fed diluted colostrum. This difference closely approximates the results of the present study, but in neither case was the difference found to be significant. It appears that no previous studies have been conducted to evaluate the effect of curd formation per se on the response of calves when tested through the entire milk-feeding period.

A definite relationship was found to exist between dilution and temperature, as well as between dilution and curding, as factors influencing weight gains and feed efficiency. When the diluted milk was fed cold, calves averaged 12 lb. more gain than when the diluted milk was warm, but when milk was fed undiluted the difference in gains between the cold and warm treatments was only 6 lb. The difference in gains between milk fed at the two temperatures was significantly greater ($P < 1\%$) when fed diluted than when fed undiluted. The TDN required to produce a pound of gain was significantly affected ($P < 5\%$) in the same manner as were gains. The reason for such differences can not be explained on the basis of this work. When feeding the diluted diets, the liquid volume consumed (in some cases over 10 lb. per feeding) exceeded the normal capacity of the abomasum. Since it is known that curdling of warm

milk occurs within 1–10 min. *(9)* after entering the stomach, curd particles may pass into the small intestine with little or no action on them by gastric secretions and may flood the intestine with more than can be readily utilized. However, cold milk might pass on, uncurdled, into the intestine before being warmed to the temperature required for coagulation (50–60° F.) *(1)*.

Dilution of the cold diets would further inhibit effective coagulation. Therefore, dilution may be of less significance in cold diets, as gastric coagulation is less likely to occur and the possibility of undissolved curd particles in the intestine is minimized. It seems that milk in the intestine as curd particles may be utilized more poorly than milk in a liquid state.

Dilution also interacted with curd formation in its effect on gains in weight and feed utilization. When diluted milk was fed citrated, gains were 13 lb. greater than when fed without the curd inhibited. The difference in gains between calves fed citrated and noncitrated diets was significantly ($P < 1\%$) greater when the milk was diluted than when undiluted. The amount of milk required to produce a pound of gain was also significantly ($P < 1\%$) affected in the same manner as gains. This interaction may also be associated with the passage of undissolved curd particles into the intestine. Diluted diets with curd-forming potential may allow passage from the stomach of undissolved curd particles; whereas, none of the other combinations of curd and dilution appears to present this possibility. Though only a speculation, this may account for the relationship discovered between curd formation and dilution.

Health and feces observations. There were no death losses during the experiment and none of the calves was observed to be adversely affected in any way by the conditions imposed. Most of the calves were in good, or very good, condition after completing the experiment. The good health of the calves in this study seems even more significant since the bedding system employed involved only the daily addition of oat straw with no removal of manure even through a postexperimental period.

The incidence of scours averaged 0.16 observation in 84 made per calf, and no cases of severe, uncontrolled scours occurred. During the experiment only four calves (each on different diets) were observed with very loose feces (scours). The average duration of this condition was less than 12 hr. per affected calf. Calves averaged approximately three observations of feces varying from normal consistency. Others *(15)* reported slight scours during the first few days following the commencement of cold milk feeding to calves two days of age. Cold skimmilk has been successfully used by Dracy and Bartle *(4)* even when fed to very young calves. Sheehy *(14)* reported that dilution was effective in alleviating and preventing digestive disorders in young milk-fed calves; however, the incidence and severity of diarrhea among calves in the control group were extremely high. Gaunya *et al. (6)* found little difference in the incidence of diarrhea between calves fed diluted and undiluted colostrum.

The absence of apparent adverse effect from feeding curd-inhibited diets is in agreement with earlier findings of this author *(12)*, but appears contrary to the conclusions of others. Blaxter and Wood *(2)* reported that if a clot

TABLE 1

Individual weight gains of calves (14–42 days of age) fed milk diets differing in physical properties

Breed	Warm milk diets				Cold milk diets			
	Undiluted		Diluted		Undiluted		Diluted	
	Curding	Noncurding	Curding	Noncurding	Curding	Noncurding	Curding	Noncurding
	(M)[a]	(F)	(F)	(M)	(F)	(M)	(M)	(F)
Holstein	(1)[b]39	(2) 45	(3) 18	(4) 24	(5) 34	(6) 20	(7) 25	(8) 48
Holstein	(1) 52	(2) 42	(3) 13	(4) 46	(5) 46	(6) 38	(7) 49	(8) 50
Jersey	(1) 20	(2) 21	(3) 15	(4) 19	(5) 24	(6) 15	(7) 11	(8) 21
Average	37	31	15	30	35	24	28	40
Average/day	1.32	1.11	0.54	1.07	1.25	0.86	1.00	1.43

[a] (M)=males; (F)=females.
[b] Diet number in parentheses.

TABLE 2

Effect of milk temperature, dilution, and curding on weight gains, feed utilization, and feces characteristics

Factor tested	Experimental period						Postexperimental period		
	14–42 days of age				14–28 days		42–56 days of age		
	No. of calves	Wt. gain	Milk intake	TDN[a]/lb gain	Feces pH	Feces moisture	Wt. gain	Milk intake	Starter consumption
		(lb/calf)	*(lb/calf)*	*(lb.)*		*(%)*	*(lb/calf)*		
Warm (T_1)	12	30	272	2.07	7.5	75	20	102	16.5
Cold (T_2)	12	32	274	2.03	7.5	73	21	108	18.6
Undiluted (D_1)	12	33	288	1.92	7.4	73	22	108	20.4
Diluted (D_2)	12	28	259	2.19	7.6	74	20	102	14.7
Curding (C_1)	12	29	264	2.13	7.3	73	19	100	17.7
Noncurding (C_2)	12	32	283	1.98	7.6	74	22	111	17.4
Standard error (main effects)		1.8		0.14	0.13	0.6	1.6		2.7
$T_1 \times C_1$	6	26	263	2.28	7.3	74	20	92	16.9
$T_1 \times C_2$	6	33	281	1.87	7.7	76	21	112	16.0
$T_2 \times C_1$	6	32	264	1.98	7.4	73	20	108	18.6
$T_2 \times C_2$	6	32	284	2.09	7.6	73	24	109	18.8
$T_1 \times D_1$	6	36	296	1.69	7.4	74	21	107	19.2
$T_1 \times D_2$	6	22	248	2.46	7.5	75	20	97	13.7
$T_2 \times D_1$	6	30	279	2.15	7.3	73	22	109	21.6
$T_2 \times D_2$	6	34	270	1.92	7.6	73	20	108	15.8
$C_1 \times D_1$	6	36	287	1.67	7.3	73	20	106	19.8
$C_1 \times D_2$	6	22	240	2.59	7.4	74	18	94	15.7
$C_2 \times D_1$	6	30	288	2.17	7.5	74	23	111	21.0
$C_2 \times D_2$	6	35	278	1.79	7.7	75	21	110	13.8
Standard error (interactions)		2.6		0.20	0.18	0.8	2.3		3.8

[a] TDN value of 20.3% used in calculations (based on Jersey milk with 5.4% fat and 14.9% total solids).

formation were inhibited or excluded, scouring inevitably followed; however, the diets used were not normal milk diets but contained gelatin or low concentrations of casein. Kastelic *et al. (7)*, using synthetic diets, concluded that coagulation of milk in the stomachs of calves is of vital importance to digestion. Scours and death resulted when young calves were fed diets in which the Ca:P ratio prevented curd formation. The satisfactory performance of a 2-wk.-old calf on the same diet was explained by the assumption that the secretion of acid and pepsin was sufficient at this age to cause coagulation of milk in the abomasum. The results of more recent work *(12)* indicates that the binding of calcium (with sodium citrate) prevents the curd from forming in calves 2 wk. of age and older. The results of the present experiment suggest that a curd formation is not needed for satisfactory performance of calves from 14 to 42 days of age.

Feces pH and moisture percentages were not significantly affected by any of the factors investigated (Table 2). Calves fed cold diets averaged slightly less moisture in the feces during the first four days on experiment than did calves on warm diets; whereas, calves fed diluted or citrated diets averaged slightly higher feces moisture than calves fed undiluted or noncitrated diets, respectively (Table 2). The higher mean feces pH for calves on citrated milk than for those on noncitrated milk may have been due to the high amount of sodium introduced into the diet with the citrate. The pH of feces averaged about 7.2 during the first days of the experiment and about 7.6 after 14 days on the study. An increase in feces pH with age has also been reported by others *(11)*. Mean pH values for individual calves ranged from 7.1 to 7.9 (with one exception averaging 5.8) and averaged 7.5 for the period of 14–21 days of age. Blaxter and Wood *(2)* reported that feces pH was lower in cases of loose feces or diarrhea; whereas, others *(11, 13)* found no difference in the pH of diarrhoeal and normal feces. These measurements on the feces, along with the gross observations, seem to affirm the absence of any tendency of the factors tested to cause diarrhea.

Postexperimental observations. During the 2 wk. subsequent to the experiment, records were kept on weight changes and starter consumption. Calves were continued on the same diets at the levels of 7 and 3% of 14-day body weights per feeding during the 7th and 8th wk. of life, respectively. Medium-quality lespedeza hay was allowed ad lib. and starter [4] was fed to a maximum of 4 lb. daily. These observations were made to determine whether the milk-feeding variable employed would influence the adjustment to consuming dry feeds. Hay intake was small and highly variable during this period.

Uniformly good growth was made during this 2-wk. period, averaging almost 1.5 lb. per day. None of the dietary factors of the milk or their interactions significantly influenced weight gains. Calves getting undiluted milk consumed nearly 40% more starter than those receiving diluted milk; however, there was

[4] Composition of starter in percentages: 24 ground corn; 22 ground oats; 29 soybean oil meal; 12 wheat bran; 10 molasses; 2 dicalcium phosphate, and 1 salt.

[a] TDN value of 20.3% used in calculations (based on Jersey milk with 5.4% fat and 14.9% total solids).

a high degree of variation in starter consumption which may, at least partially, account for this large difference lacking statistical significance (Table 2).

The foregoing studies indicate that the physical properties of the diet may be as important as the nutrients themselves in the utilization of the milk diet for growth. Since dilution has been found to be related both to temperature and to curd formation in the response of calves, it would appear that the nutrient concentration of liquid diets deserves more intensive investigation.

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FACTORS ASSOCIATED WITH ESTIMATED FERTILIZATION AND SERVICE EFFICIENCY OF COWS [1,2]

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SUMMARY

Embryonic mortality was estimated by using the difference between percentage of estrous cycles recurring between 18 and 25 days for the cows which were bred at the previous estrus compared with those which were not bred. The study included 6,719 calvings carried to term, 668 abortions, and 1,915 estimated embryos lost, for a total of 9,302 fertilizations. The estimated embryonic loss for all cows was 20.6% and abortion loss was 7.2%. Clinically normal cows averaged 11.4% and clinically normal cows and heifers averaged 16.6% embryonic loss. Though fertilizing capacity was equal, normal heifers had a significantly higher embryo loss than cows in their second and third reproductive periods. Fourth and fifth reproductive period cows had the highest efficiency as determined from the percentage of services carried to term. Fertilizations per service and embryo loss declined markedly with increasing age. Reproductive efficiency was very low for cows with infections of the genital tract and estimated embryo loss exceeded 40%. Losses were equally high for cows with cystic ovaries. Cows having only short estrous cycles averaged 28.8% embryo loss and 24.2% services carried to term. Cows with an abnormal calving from the preceding gestation averaged 14.4% embryo loss and 39.2% services carried to term. Monthly differences in reproductive efficiency were relatively unimportant as compared with yearly differences.

In 1914, Hammond *(10)* recognized that prenatal death was common in livestock. Prenatal death is now recognized as a major cause of infertility. The basic causes are poorly understood, but variable rates have been associated with a variety of environmental and physiological factors.

Cows returning in estrus after breeding generally show more irregularly long cycles than unbred cows *(5, 13)*. When estrous cycle distributions are known for enough cows before breeding, the change among those repeating for service offers a practical way to estimate the probable embryonic death. It was the purpose of this study to estimate changes in reproductive efficiency associated with clinical condition and age of cow and month and year of fertilization.

MATERIALS AND METHODS

The breeding records for this study covered a 30-yr. period (1920–50) and involved 9,994 breeding periods of 2,607 Holstein-Friesian cows at Carnation Milk Farms, Carnation, Washington. Greater detail concerning general aspects of the data have been previously published *(5, 18)*. Three different veterinarians, in residence during the study period, personally recorded results of all clinical

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[2] The authors express acknowledgment to Carnation Milk Farms, Carnation, Washington, for making available the data used.

examinations on individual cow-history cards. After 1925, one herdsman was responsible for all nonclinical breeding records. These included all estrous dates, in addition to other breeding records maintained by progressive purebred herd owners.

Methods of housing and general management were uniform during the study period. All cows could be readily observed during the day, whether on pasture, in box stalls, or in exercise pens. While observation at night was not ideal, some cows in the herd were milked four times daily throughout this period; all were milked at least thrice daily. This reduced the time cows were not under observation.

Natural service was used before 1940. Artificial service with semen containing no antibiotics was used exclusively after 1940. Estrus occurring after conception was omitted from the present study. Erb *et al. (5)* compared the 28,639 estrous cycles recorded for this herd with other similar studies, and concluded that the estrous cycle distributions were comparable with those of other cow populations. These data *(5)* were adapted to the present study.

The following data were available for the various subclasses of cows under study: *(a)* Total services; *(b)* total conceptions—defined in terms of total calvings and observed abortions; *(c)* total services (including services to cows not again calving) for conceptions as defined in *(b)*, and *(d)* distribution of estrous cycles for each subclass of cows not bred at the previous estrus (nonservice cycles) and those which repeated for service (service cycles).

From these data the following estimates were made: *(e)* number of repeat services for each subclass of cows *(a-b)*; *(f)* percentage difference in number of 18- to 25-day cycles between service and nonservice cycles after removing differences due to numbers of cycles 2–17 days in length by setting the nonservice cycle rate equal to the observed service cycle rate. Under these conditions, per cent 18–25 day nonservice cycles − per cent 18–25 service cycles = per cent service cycles over 25 days − per cent nonservice cycles over 25 days. *(g)* Estimated embryonic loss ($e \times f$); *(h)* total fertilizations [term calvings + abortions + estimated embryonic loss *(g)*]; *(i)* fertilizations/service; *(j)* per cent embryonic loss (number estimated/total fertilizations); *(k)* per cent abortions (number observed/total fertilizations); *(l)* per cent fertilizations carried to term [100% − $(j + k)$]; and *(m)* per cent total services carried to term [$h - (g +$ abortions)/ total services].

Calculation *(i)* estimates the service efficiency in terms of total fertilizations; whereas, *(l)* estimates the percentage of fertilizations carried a full gestation, and *(m)* represents the percentage of calves carried to term on a total service basis.

An example of the above procedure is presented for clarification. Take a subclass of cows involving 1,000 total services and 380 term calving and 20 abortions. In this subclass (1,000–400), 600 repeat services were involved. Fifty per cent of the service cycles occurred within 25 days after breeding, as compared with 60% for the same cows when not bred at a previous estrus (nonservice cycles). The difference is 10%. Then 10%, or 60 of the 600 repeat services, are estimated fertilizations with subsequent embryo loss. Therefore, the original

1,000 services resulted in (380 + 20 + 60), or 460 total fertilizations. The service efficiency is 460/1,000, or 46%. The estimated embryo loss is 60/460, or 13.0%. The abortion rate is 20/460, or 4.8%. The fertilizations carried to term are 100% − (13.0 embryo loss + 4.8% abortions), or 82.2%. The services carried to term are 380/1,000, or 38%.

The 1 and 5% confidence limits *(20)* were used as a practical test for significant differences.

RESULTS AND DISCUSSION

The results are reported for 7,387 calvings. Of these, 668 were observed abortions. There were 22,456 total services; 15,069 were terminated by a subsequent estrus.

There were 59.005% of the nonservice and 46.3% of the service cycles falling within the 18–25 day interval. The estimated embryonic mortality for all cows was 1,915 [15,069 × (59.005% − 46.300%)]. These were added to the total of 7,387 calvings, making an estimated 9,302 fertilizations.

Table 1 shows that the estimated embryonic death rate was (1,915/9,302), 20.6%. Hawk *et al. (13)*, using a similar method, estimated embryonic mortality by 150 days at 19.9% for 837 services. In addition, there were (668/9,302), 7.2% observed abortions, bringing the total fertilizations lost before term to 27.8%.

Normal and Abnormal Cows

Normals. Table 1 shows estimates for cows in various reproductive states. Clinically normal reproductive periods (Groups I) were those commencing with the birth of a living single calf. The cow may have had no more than one estrous cycle less than 16 days and no other symptoms of clinically abnormal reproductive function. Compared with the herd average, this group was distinctly superior, requiring fewer services per fertilization with over one-half of the services carried to term. The estimated embryonic mortality was only 11.4%; the herd average was 20.6%. As will be shown later, heifers had a higher embryonic death rate than older cows. When clinically normal heifers were included with clinically normal cows (Group II), fertilization efficiency was decreased and the estimated embryonic mortality was 16.6%.

Abnormals. The respective subclasses of reproductively abnormal cows showed serious impairment of subsequent reproduction through reduced fertilizing capacity and increased mortality of fertilizations which occurred. Subsequent reproduction was least affected by abnormal calvings. However, as compared with Group I (no heifers involved) there was a significant decrease in fertilizations per service (P 0.001) and fertilizations (P 0.01) and services (P 0.001) carried to term. Estimated embryonic mortality and abortions increased slightly (P 0.10).

The remaining abnormal subclasses were associated with highly significant changes in reproductive efficiency as compared with Group II cows and heifers, or with cows starting reproductive periods with an abnormal calving.

Reproductive periods having two or more short estrous cycles showed considerably more reduction in over-all reproductive efficiency than previous data

TABLE 1

Estimated embryonic and service loss among cows with different clinical indications of reproductive disorders

Cow	Estimated fertilizations		Nonreturns at 25 days—returning for service by Day				Observed abortions	Carried to term	
	Total	Efficiency	45	65	100	Term		Fertilization	Services
Group	(No.)	(%)							
Study total	9,302	41.5	7.3	12.2	16.6	20.6	7.2	72.2	29.9
Clinically normal cows (Group I)	3,252	62.9	4.2	7.3	9.7	11.4	5.9	82.7	51.9
Clinically normal cows + normal heifers (Group II)	5,409	56.8	7.3	11.8	15.0	16.6	6.6	76.8	43.8
Abnormal									
Starting with abnormal calving only *(a)*	1,075	50.3	3.7	5.9	11.8	14.1	8.0	77.9	39.2
Only short cycles (all types of calvings)	981	38.0	10.4	17.2	24.4	28.8	7.5	63.7	24.2
Cystic follicles or retained corpora lutea *(b)*	539	26.3	0	32.4	35.9	44.2	6.7	49.1	11.3
Vaginitis *(c) (d)*	506	40.8	25.6	37.0	41.5	45.5	4.3	50.2	20.5
Cloudy mucus *(c) (e)*	556	26.7	9.0	20.3	37.8	41.1	5.9	53.0	14.0
Uterus abnormal *(c) (f)*	735	34.4	0	10.6	37.0	49.2	6.8	44.0	15.2
Total for infection *(c) (g)*	1,593	32.1	17.7	30.3	40.0	46.4	6.5	47.1	15.1

[a] Includes stillborn single births, twins, abortions, and retained placentae.
[b] Includes when in combination with all other abnormalities.
[c] Includes combinations with short estrous cycles, cystic follicles, and retained corpora lutea on ovary.
[d] No other pathologic condition observed.
[e] Includes combinations with vaginitis and other pathologic conditions, except cervical and uterine infection.
[f] May be in combination with any pathogenic condition—includes metritis, poor tone, and enlargements.
[g] Totals for infected cervix and uterus, cloudy mucus, and vaginitis.

have indicated. Erb and Ehlers *(4)* have previously shown from these data that significantly more services were required for conception when normal cows were bred following cycles 2–17 days in length. Since less than 8% of the total services which failed during reproductive periods with only short estrous cycles were 2–17 days in length, it was likely that conditions predisposing embryonic mortality were present at other times. Further evidence of this is shown (Table 1) for cystic conditions of the ovary (71% were cystic follicles and 29% were retained corpora lutea). The estimated embryonic mortality was 44.2%. This latter group may have been in combination with other reproductive tract abnormalities and, thus, contains more biasing factors than the short estrous cycle group.

Of the pathogenic conditions, vaginitis showed the least interference with subsequent reproduction, although, compared with Group II, the estimated embryonic death was nearly threefold higher and four services out of five failed to carry to term. A peculiarity of this subclass was that 25.6% of the embryonic deaths was indicated by return to estrus within 45 days after breeding. The vaginitis total also includes 27% with short estrous cycles and 3% with cystic conditions of the ovary. However, the high percentage of returns by 45 days was a peculiarity of vaginitis, since only 10.4% of the embryonic deaths was indicated by 45 days for the short estrous cycle group and none for the cystic group. Oddly, this latter group had 32.4% of the total of 44.2% of embryonic deaths indicated between 46 and 65 days, and the abnormal uterus group had a maximum rate from 66–100 days. Part, but not all, of the differences may be attributable to age, because the older cows had a higher proportion of long return-to-service intervals ("). Vaginitis was most common during the first two breeding periods. Short estrous cycles uncomplicated by other abnormal conditions also occurred more frequently in younger cows.

It would have been desirable to further separate cystic tendency and infections of the reproductive tract. However, considerable data were necessary to estimate differences in distribution of nonservice and service cycles for each subclass of cows. From a practical standpoint, it is evident that infections were a primary cause of lowered fertility and of high embryonic loss. Cystic tendency was three times more frequent among cows with other abnormal pathology than the herd average. Cows that showed clinical evidence of abnormal reproductive function usually were not bred. Therefore, it is conceivable that the uterus is physiologically as well as pathogenically incapable of supporting pregnancy for variable periods after clinical symptoms are no longer observed. Erb and Morrison *(6)* have shown that estrus after conception was more frequent subsequent to observation of cystic follicles on the ovary. The cause of this phenomenon is unknown, but endocrine imbalances are indicated.

Age

Reproductive periods were divided into clinically normal and abnormal groups. These were further subdivided into calving periods (as shown in Table 2). Clinically normal heifers (Table 2) apparently fertilized as efficiently as normal

TABLE 2

Estimated embryonic and service loss among clinically normal and abnormal cows in different reproductive periods

Reproductive period of cows	Estimated fertilizations		Nonreturns at 25 days—returning for service by Day			Term	Observed abortions	Carried to term	
	Total	Efficiency	45	65	100			Fertilization	Services
	(No.)	*(%)*							
				Clinically normal [a]					
1	2,207	51.3	15.0	22.1	25.7	26.1	7.0	66.9	34.3
2, 3	2,264	52.4	8.6	14.7	19.2	22.1	6.9	71.0	37.2
4, 5	1,167	46.7	0.8	1.1	5.2	10.4	6.0	83.6	39.0
6, 7	643	42.0	0	3.8	9.1	9.8	5.1	85.1	35.7
8, 9	330	34.2	0	0	7.8	13.3	6.1	80.6	27.6
>9	131	31.3	0	0	0	1.5	7.6	90.9	28.4
				Clinically abnormal					
1	482	30.4	22.8	29.2	36.9	43.2	7.7	49.1	14.9
2, 3	1,112	32.9	16.1	23.8	29.2	37.1	7.4	55.5	18.3
4, 5	617	24.1	5.6	9.8	13.4	23.4	8.8	67.8	16.4
6, 7	375	22.9	0	10.5	22.4	28.2	6.9	64.9	14.8
8, 9	163	17.6	0	0	0	5.5	10.4	84.1	14.8
>9	197	22.5	12.2	32.7	52.6	58.2	3.6	38.2	8.6

[a] Normalcy of calving not considered.

cows in their second and third reproductive periods (51.3 vs. 52.4% fertilizations per service). Similarly compared, normal heifers had a significantly greater total embryonic loss (P 0.05) and fewer fertilizations (P 0.05) and services carried to term (P 0.10).

Normal cows in their fourth and fifth reproductive periods had the highest percentage of services carried to term because of a marked decline in the estimated embryonic loss as compared with the younger cows (P 0.001), and higher fertilizing capacity than older cows. Fourth and fifth reproductive period cows carried significantly more services to term than cows in their eighth and ninth (P 0.01) and over ninth reproductive periods ((P 0.05). Fertilizations carried to term and estimated embryonic death changed inconsistently as the reproductive period of the cows increased above five; however, fertilizations per service declined from 46.7% for the fourth and fifth reproductive periods down to 31.3% for the oldest group of cows. Three prominent features, associated with increasing age, shown in Table 2, are *(1)* the decrease in estimated embryonic mortality; *(2)* the decrease in ability to fertilize; and *(3)* lesser over-all differences in services carried to term. A fourth feature is that younger cows returned for service sooner after an apparent embryonic loss. Erb *et al. (5)* had earlier found that heifers cycled more regularly and had proportionately fewer non-service cycles over 25 days long. The heifers had significantly more 26- to 35-day cycles following service than did older cows *(5)*. These differences are indicated in the returns to service, reflecting embryonic loss at intervals from 26–35 to over 100 days (Table 2).

Similar data for abnormal heifers and cows are shown (in the lower portion of Table 2). Changes with age were similar, except that reproductive efficiency was much lower for all measurements used.

The finding that heifers and second and third reproductive period cows have higher embryonic mortality is not opposed to the findings of Tanabe and Casida *(22)* and Tanabe and Almquist *(21)*, because in these experiments clinically normal repeat breeder cows and heifers were used.

Months and Years

Erb *et al. (5)* previously found from these data that yearly variation in percentage of the total estrous cycles falling within an 18- to 25-day interval was approximately nine times greater than variation attributable to months. Estimates of reproductive efficiency are shown for all cows by months (in Figure 1) and by years (in Figure 2). Monthly variations were relatively unimportant as compared with years. In interpreting seasonal variations, it is necessary to consider geographical locations *(7)*, because high environmental temperatures appear a factor in some areas and not in others. Estimated embryonic mortality (P 0.001) and abortions (P 0.10) were higher from July through December, but percentage of services carried to term did not vary significantly.

Yearly variations were evident for all the measures of reproductive efficiency shown in Figure 2. Abortion rates were high during the period when the herd was infected with brucellosis (1926–32), and reached a second peak in 1940. This

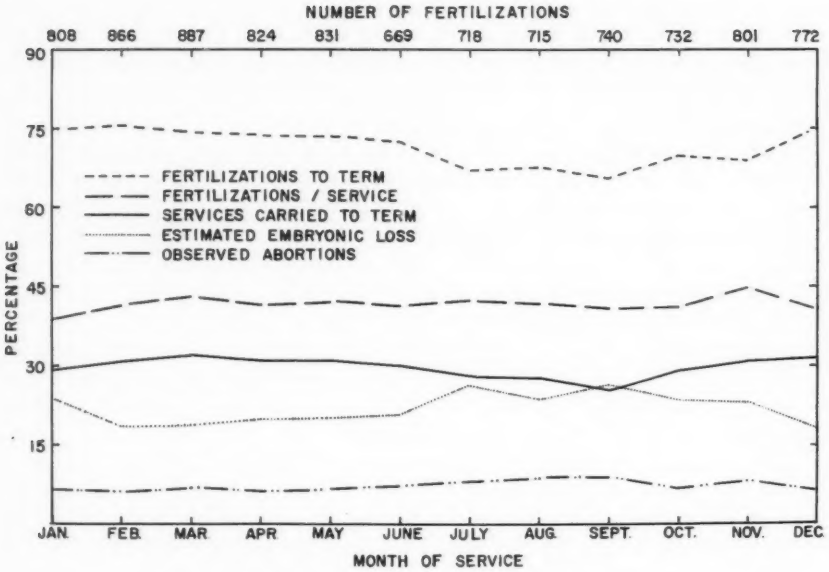

FIG. 1. Estimated embryonic and service loss by months.

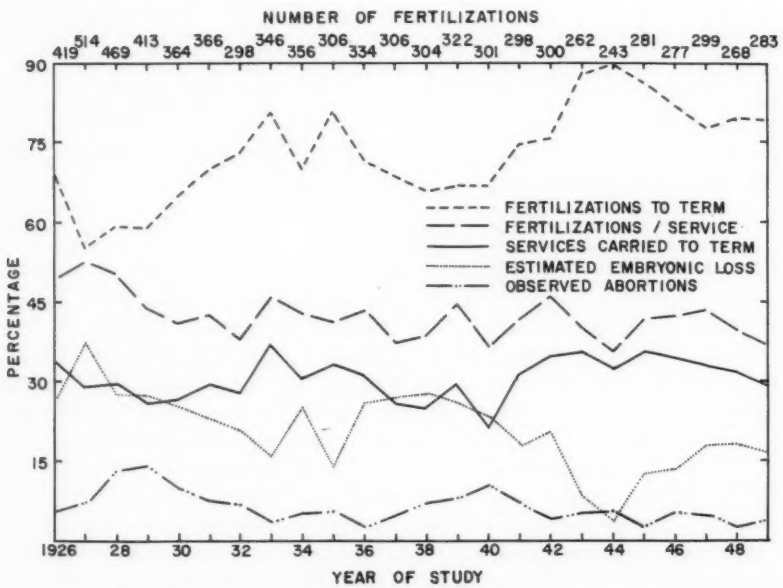
NUMBER OF FERTILIZATIONS
419 514 469 413 364 366 298 346 356 306 334 306 304 322 301 298 300 262 243 281 277 299 268 283
90
75
60
45
30
15
PERCENTAGE
FERTILIZATIONS TO TERM
FERTILIZATIONS / SERVICE
SERVICES CARRIED TO TERM
ESTIMATED EMBRYONIC LOSS
OBSERVED ABORTIONS
1926 28 30 32 34 36 38 40 42 44 46 48
YEAR OF STUDY

FIG. 2. Estimated embryonic and service loss by years of the study.

latter peak was likely due to a mild outbreak of trichomoniasis or, possibly, vibriosis. Artificial insemination was used after 1939. Estimated embryonic loss was generally lower after 1942. This coincided with lower abortion rates and a higher percentage of the fertilizations carried to term. Proportionately more artificial services apparently failed to fertilize, because estimated fertilizations per service showed no consistent trends after 1929, though the percentage of services carried to term remained at a consistently higher level (less year-to-year differences) after 1941. Morrison and Erb *(18)* have reported services required for conception for the normal cows. Though year-to-year variation was present, the services/conception ratio did not change. The results (in Figure 2) for all cows indicate the inadequacy of a single measure of reproductive efficiency. Pathogenic environment changed quite drastically during the study period, because brucellosis was prevalent before 1932 and vaginitis was a major problem during the last 10 yr. of natural breeding. Losses from abortion and embryo death reflect these changes.

DISCUSSION

Embryonic death rates by 34 days post-fertilization have been shown to be very high among clinically normal repeat breeder cows *(22)* and heifers *(21)*. Evidently, most of this loss occurs between 16 and 34 days *(14)*. Comparisons using virgin heifers and semen from high- and low-fertility bulls indicate that one major difference is fertilization rate *(1, 15)*. However, in one study *(1)* the embryonic losses were 10.5 and 19.6%, respectively, following breeding with semen from bulls of high and low fertility. Therefore, over-all reproductive efficiency may be quite different, depending on factors influencing *(a)* fertilization rate, *(b)* embryonic loss, *(c)* fetal losses from abortion, and *(d)* calves dead at birth.

The question may be raised that 7,387 calvings from 22,456 services are hardly typical. However, nearly 4,000 of these services were to cows which did not conceive before leaving the herd, a group generally omitted in studies of female reproductive rates. Purebred herd data can be expected to show lower efficiencies than the average dairy herd, because greater emphasis is placed on progeny from favored cows and bulls and the owner can afford to retain animals longer, even though fertility is very low because of injury, illness, and senility. Even under these conditions, clinically normal heifers and cows (Group II—Table 1) had a services-per-conception efficiency of 50.4%.

Compared with 70% for 60- to 90-day nonreturns to first service in cows bred artificially, 50% seems low. However, when one remembers that late returns to service, death of the cow after breeding, slaughter after a late return, and repeat services are not considered in such data, the real reproductive efficiency is considerably less. Such data are hardly appropriate for direct comparison with the present study.

Using only first services in a study of this type was considered wholly inappropriate, since the emphasis was on service efficiency as influenced by a variety of environmental factors. Changes in reproductive efficiency during years of the study (Figure 2) show that different pathogenic and physiological

factors were involved at different times, and that these were well-distributed throughout months of the year, since seasonal variations were relatively unimportant (Figure 1).

It is realized that not all abnormal reproductive periods of the cows were clinically recognized, since not all cows were rectally palpated. Fitness for first breeding was largely determined by regular estrus and clear mucous discharge. Many questionable cows were examined and considered normal. One can expect that many of the cows classified as clinically normal (in Table 1) were afflicted with subclinical disorders. Rasbech *(19)*, Gunter *et al. (9)*, and Lindley and Hatfield *(16)* have found indications of subclinical infection in a high percentage of clinically normal cows, as well as in infertile cows *(9, 16)*.

For comparative purposes, very little bias can be expected, because appearance of unrecognized abnormal cows among the normal groupings would be random. The subgroups of abnormal cows were diagnosed by highly competent resident veterinarians, or recognized by keen observation (abnormal births, short estrous cycles, and discolored mucus).

In any study of this type, two major factors would be operating at greater or lesser intensity, depending on the particular population of cows involved: *(a)* Differences in fertility level of bulls and *(b)* differences in fertilizing level of the cows. Each of these likely would be influenced largely by pathological environment, resulting in a range from comparatively high fertilizing capacity and high embryonic loss to a low fertilizing rate and low embryonic loss. Embryonic loss would almost automatically be lower in the latter case, because of relatively fewer fertilizations. Semen of low-fertility bulls *(3, 8)* affects the fertility of cows repeating for service, as compared with cows repeating for service subsequent to breeding with semen from high-fertility bulls. The clinically normal cows averaged 62.9% fertilizing efficiency and 51.9% of the services carried to term, as compared with 41.5 and 29.0% for all cows and heifers in the study. The embryonic loss was estimated at 11.4% for the normal cows and 20.6% for all cows and heifers. Experimental conditions of other studies have been different. Kidder *et al. (15)* estimated that in terms of first services in artificial breeding, 40.4% were still pregnant at 150 days post-breeding. Because most observed abortions would occur after this time, the discrepancy between the estimate of Kidder *et al. (15)* and the present study would be less than 40.4% vs. 29.9%. Two other differences are involved: *(a)* Kidder *et al. (15)* considered only first-service cows and *(b)* the bulls were in commercial artificial breeding, thus giving a higher and lesser range in fertilizing capacity of the semen.

The evidence that fertilizing capacity was a primary difference is good, since estimates of embryonic loss are in good agreement. Hawk *et al. (13)* reported 19.9% embryonic loss by 150 days post-breeding. Kidder *et al. (15)* estimated 20% by 60–90 days, based on discrepancy between three-day fertilizations and 60–90 day nonreturns. These estimates are comparable with the 20.6% for the present study, which was based on the discrepancy between the percentage of cycles recurring in 18–25 days for cows bred and not bred at the previous estrus.

Interference with reproductive efficiency reached serious proportions in cows

diagnosed with infections of the reproductive tract or having cystic ovaries. Fertilizing capacity was much lower, and those which fertilized had estimated embryonic losses exceeding 40% (Table 1). Indications are that abnormal physiological and pathogenic conditions are separately involved, because cows and heifers showing only short estrous cycles had an estimated embryonic loss of 28.8% as compared with 16.6% for normal cows and heifers (Table 1). Further evidence that physiologic factors are involved has been shown in the several studies using repeat breeder cows and heifers *(12, 14, 21, 22)*. Hawk *et al. (12)*, using clinically normal repeat breeders, observed that only 11.6% had culturable bacteria in the cervix and the uterus, and concluded that infection was not a major factor among cows of this type.

The association of high embryonic loss with reproductive tract infections is interesting, considering the evidence of Black *et al. (2)* and McDonald *et al. (17)*. They have presented evidence indicating that the luteal phase uterus is more vulnerable to inflammatory reactions than is the follicular phase uterus. They also report that repeat breeder luteal phase cows did not show the same reaction as luteal phase virgin heifers *(2)*. Apparently, management practices designed to rigidly control infectious agents would result in the greatest improvement in reproductive efficiency. Under these conditions, cystic tendency may be greatly reduced, if for no other reason than that cows would conceive sooner and thus reduce the time for endocrine malfunction. Even if cystic tendency increased the vulnerability of the reproductive tract to infection, this could hardly occur if a minimum number of infective organisms was present.

High embryonic losses among clinically normal young cows as compared with older cows adds a further statistical problem to studies designed to elucidate possible genetic and environmental causes of embryonic loss.

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EFFECT OF METHOD OF GLYCEROL ADDITION ON THE FERTILITY OF FROZEN BOVINE SPERMATOZOA [1]

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SUMMARY

An experiment was conducted to determine the optimum method of adding glycerol to bovine spermatozoa for freezing. Two separate semen collections were taken from six different bulls. A total of 3,005 first-service cows were bred. Each semen collection was used on a split-sample basis. Four methods of glycerolization were employed; namely, (A) addition of the glycerolated buffer to the nonglycerolated semen portion at 5° C., (B) at 10° C., (C) at 20° C., and (D) addition of freshly collected semen directly to the total glycerolated buffer. Respectively, a total of 770, 742, 723, and 770 first-service cows were bred. The nonreturn rates based on 75-day nonreturns were 65.1% when the glycerol portion was added at 5° C., 65.4% when added at 10° C., 62.7% when added at 20° C., and 64.0% when the semen was added directly to the total volume of glycerolated buffer.

By analysis of variance there were no significant differences in fertility among the four tested treatments, weeks of storage, collection periods, or their interactions. There was a significant difference in fertility among bulls, but this difference was not influenced by treatment or length of storage. A positive but nonsignificant correlation was found between fertility rate and estimate of percentage motility of the spermatozoa after thawing.

Since the freezing of bovine spermatozoa is a relatively new process, many details concerning procedure must be studied to determine satisfactory methods which will result in maximum fertility upon thawing following freezing. The most suitable method of adding the protective agent, glycerol, is one of the first steps that must be determined.

Polge and Rowson *(8)* were unsuccessful in obtaining conception with frozen bovine semen when the semen was added directly to a glycerolated citrate buffer containing 50% yolk at 30° C. Revival following thawing was reported to have been poor. It was not until the extended semen was allowed to cool to 5° C. over a 4-hr. period, and the glycerol then added in an equal portion of extender at the same temperature, that satisfactory conception rates were obtained. Polge *(7)* reported that spermatozoa revival rate was much higher when glycerol was added at 5° C. than at 20° C. However, Holt *(4)* indicated that glycerol could be added as soon as ½ hr. after collection of the semen and at room temperature. Revival obtained using this method was comparable to results obtained when glycerol was added to semen that had been cooled to 5° C. Dunn and Hafs *(1)* obtained revival rates which were higher when the semen was glycerolated at 5° C. than at room temperature. The difference, however, was not statistically significant.

Miller and VanDemark *(5)* stated that adding glycerol to semen diluted with yolk citrate at 4–5° C. gave slightly higher results than adding it at either 10 or

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15.5° C. They also suggested that the addition of the glycerol solution in three equal portions at 10-min. intervals resulted in slightly higher sperm survival after freezing than did a complete addition at one time. O'Dell and Almquist *(6)* reported that superior revival and survival rates were obtained with heated milk extenders when the glycerol was added in five equal portions at intervals of either 6 or 12 min., rather than adding the glycerol portion either in three equal parts at 10-min. intervals or all at once.

Vogt and Graham *(9)* found no difference in percentage of spermatozoa revival after freezing, when glycerol was added to the extended semen portion, at 30, 20, 15, 5, or 2° C. They also found no significant difference in survival rate of the above methods or by adding the raw semen directly to the glycerolated buffer at 35° C.

Due to the fact that little uniformity exists among the results obtained by various research workers, this study was undertaken to establish the optimum method of glycerol addition.

MATERIALS AND METHODS

In this study, semen was collected from mature Holstein bulls selected at random from six different artificial breeding organizations. Semen was collected by the use of an artificial vagina as a special collection. In all cases, a mixture of the first and second ejaculates was used for freezing. Semen from all bulls was frozen in a yolk-citrate medium containing a final concentration of 7% glycerol, 2.02% trisodium citrate (dihydrate), and 23.25% egg yolk. The antibiotics, penicillin (potassium crystalline G) and dihydrostreptomycin sulfate, were incorporated into the extender at a rate of 1,000 units and 1,000 μg. per milliliter, respectively *(2)*. A final semen extension rate of 1:50 was employed.

Only four of the variations tested by Vogt and Graham *(9)* were compared in this fertility study. They were the addition of glycerolated extender in three equal portions, which constituted 75% of the total extender at temperatures of (A) 5° C., (B) 10° C., (C) 20° C., and (D) addition of freshly collected semen directly to the total glycerolated extender at approximately 35° C. After the addition of glycerol, Samples (B), (C), and (D) were cooled from the temperature of addition of the glycerol solution at the rate of 0.5° C. per minute to 5° C.

All samples were allowed to equilibrate 12 hr. from the time the last glycerolated portion was added and 12 hr. from the time the raw semen was added directly to the glycerolated buffer in the case of sample (D). This is in accord with the findings of Graham *et al. (3)*.

The rate of freezing in all cases was 3° C. per minute from 5 to −15° C., and 5° C. per minute from −15 to −79° C. These rates were based on the results found to be optimum by Erickson *et al. (2)*. The semen samples were stored at −79° C. until used.

Field trials were conducted in two parts, representing two different semen collections from each of the six bulls. Two samples of frozen semen of each variable were sent to each of 63 technicians after 1, 2, 3, and 4 wk. of storage for each collection period. The technicians selected the semen samples at random

from their field-kit and used them on the first eight first-service cows of the breed. Samples were thawed at approximately 5° C. After each insemination, the technician wrote down on I.B.M. cards the necessary data to identify the cow, semen sample, date of service, collection period, and week of storage. The nonreturn rates were compiled from the I.B.M. cards based on 75-day nonreturns, which is comparable to a 60- to 90-day nonreturn rate.

RESULTS AND DISCUSSION

The sources of variance in the experimental design were analyzed by analysis of variance. Differences among treatments, replicates (collection periods), weeks of storage, and associated interactions were not significant. A significant difference was noted among bulls. The differences among bulls were not significantly influenced, however, by treatment or length of storage.

The summary of the percentages of nonreturns for treatments by bulls is given (Table 1). Little variation between treatments within bulls was found. Bulls H-3 and H-5 were consistently low in percentage of nonreturns, irrespective of treatment; whereas, bulls H-1 and H-2 were consistently high. Of the two bulls (H-3 and H-5) that were consistently low as to percentage nonreturns, it is interesting to note that in each case a higher nonreturn rate was obtained when the treatment consisted of adding the raw semen directly to the glycerolated buffer.

TABLE 1

Nonreturn rates for methods and temperatures of glycerol addition

	Conventional 4° C.		10° C.		20° C.		Direct	
Bull	First services	Nonreturn 75-days	First services	Nonreturn 75-days	First services	Nonreturn 75-days	First services	Non-return 75-days
	(No.)	*(%)*	*(No.)*	*(%)*	*(No.)*	*(%)*	*(No.)*	*(%)*
H-1	149	68.5	135	74.8	158	77.2	153	74.5
H-2	45	73.3	45	73.3	45	71.1	46	71.7
H-3	121	55.4	109	57.8	115	49.6	123	63.4
H-4	166	72.3	169	63.3	176	67.6	166	63.9
H-5	117	59.0	101	57.4	95	53.7	115	62.6
H-6	172	64.0	179	67.0	134	53.7	167	53.9
Total	770	65.1	742	65.4	723	62.7	770	64.0

The difference between 1, 2, 3, and 4-wk. storage (Table 2) was not significant. By analysis of variance there were no significant differences in fertility due to the interaction between methods of glycerol and length of storage. Again, it is of interest to note that there was no downward trend as storage time increased but, rather, an upward trend. Although the difference is nonsignificant, the upward trend in percentage of nonreturn rates may, in part, be attributed to the fact that as the experiment continued the technicians were becoming more familiar with the techniques involved in the handling of frozen semen.

Correlation between motility rating and conception rate was determined, using the average progressive motility rating and corresponding nonreturn rate

TABLE 2

Nonreturn rates for storage time interval by method and temperature of glycerol addition

Treatment	Week 1		Week 2		Week 3		Week 4	
	First services	Nonreturn 75-days	First services	Nonreturn 75-days	First services	Nonreturn 75-days	First services	Nonreturn 75-days
	(No.)	*(%)*	*(No.)*	*(%)*	*(No.)*	*(%)*	*(No.)*	*(%)*
4° C.	203	66.0	203	66.0	194	68.0	170	59.4
10° C.	199	66.8	195	58.5	179	64.8	169	72.2
20° C.	198	60.1	194	63.9	185	62.7	146	64.4
Direct	210	61.4	199	60.8	188	62.8	173	72.3
Total	810	63.6	791	62.3	746	64.6	658	67.1

for each treatment, weeks of storage, and collection period for each bull. The range in motility ratings was between 34.2 and 61.5%, with an over-all average of 53.1%. A positive correlation of percentage progressive motility and nonreturn rate based on 75-day nonreturns of 0.251 was not significant at the 5% level.

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EFFICACY OF OAT HAY AS A LEGUME BLOAT PREVENTIVE IN CATTLE

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SUMMARY

Excellent quality oat hay was fed overnight to 17 dry cows, to study its effectiveness in controlling bloat resulting from ingestion of succulent, immature alfalfa pasture. The experiment was divided into the pre-test, test, and post-test periods. Each period was divided into four morning and two afternoon grazing subperiods. It was found that the overnight feeding of an average of 12 lb. of oat hay per cow significantly reduced the incidence and severity of acute legume bloat.

The mechanism by which grass hays offer protection from bloat is unknown; however, three possibilities have been suggested: *(1)* They physically prevent the formation of froth, *(2)* they contain a chemical antifrothing agent, or *(3)* their scabrous nature causes a stimulation of receptors in the rumen responsible for maintaining the tonic activity of rumen motility and eructation by reflex action. Perhaps all three factors act simultaneously.

The effectiveness of Sudan grass hay and pasture in the prevention of acute legume bloat in cattle was demonstrated by Cole, Mead, and Regan *(5)* and Cole and Kleiber *(3)*. It was necessary for the animals to consume at least 17 lb. of Sudan grass hay the night before grazing succulent, immature alfalfa pasture, if complete protection from bloat was to be obtained. Attempts to prevent bloat with barley straw were unsuccessful *(19)*, presumably because it was so unpalatable that only small amounts were consumed.

Recently, Johns *(15)* was unsuccessful in preventing acute legume bloat in dairy cattle prefed up to 2 lb. of "hay" per head. Hogg and Barrentine *(12)* prefed 5 lb. of "hay" to cattle before they were grazed on Ladino clover and found a reduction in bloat in the morning but not in the afternoon. Neither of these workers indicated the kind of hay employed. On the other hand, Meyer and coworkers *(21)* and Hull *et al. (13)* have achieved satisfactory control of legume bloat by following the recommendations of Cole and coworkers *(3, 5)*.

This study was undertaken to determine whether another grass hay could be substituted for Sudan grass hay for acute legume bloat control. Oat hay was chosen because it is highly palatable to cattle and is readily available to many livestock enterprises.

EXPERIMENTAL PROCEDURE

Relatively pure stands of alfalfa were grazed in these trials to produce bloat. Electric fences were placed 10 ft. inside all existing fences, to eliminate the consumption of weeds and grasses. The pasture was divided in half, with

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one half being cut ten days previously to the other so that immature plants would always be available. The initial grazing was begun when the pasture was between 12 and 16 in. high, with no evidence of bloom. At this stage, the tops of the plants were succulent and turgid and snapped when broken with the fingers. Grazing was continued until the tops of the plants were no longer succulent or when bloom became evident.

Dry cows from the station herd, 15 Jerseys and two Holsteins, were used as experimental animals. Some had a history of bloating, but others were chosen at random. Seventeen cows were used in the study, excepting for one day, when 16 were used. When not on pasture, all cows were kept in a nearby dry lot, where they had free access to water but not to feed.

Before the first day of alfalfa grazing, the cows were fasted for 12 hr. When permitted to graze for the first time, all types of vegetation other than alfalfa were eaten first. By the second day, the cows were hungry and the few contaminating plants were gone, so the alfalfa was consumed eagerly.

All animals served as their own controls, inasmuch as they were pastured without oat hay both before and after the test period in which the efficiency of oat hay was determined. By this means, it was demonstrated that the animals would bloat on the pastures used when oat hay was not provided.

The grazing day was divided into two portions, morning and afternoon. In the morning, the cows were placed on the pasture at an average time of 8:50 A.M. and removed at 11:45 A.M. From 11:45 A.M. until 1:35 P.M., they were placed in the holding corral, where they had free access to water. At 1:35 P.M., grazing was again permitted and continued until 4:50 P.M., at which time they were returned to the holding corral. Such a procedure resulted in an approximate 3-hr. grazing period in the morning and in the afternoon.

Each period of the experiment consisted of four days. During the test period, excellent quality oat hay was fed free choice overnight. The amount of hay eaten was recorded for all 17 animals as a group, not for individuals.

During the time the cows were on pasture, they were under constant supervision. After bloat had begun to develop, tympanometer (*17*) readings were made continually on all animals until the bloat began to recede. Several readings were taken consecutively on each animal and the average of these was taken as the value for that time.

The degree of bloat observed has been arbitrarily divided into several categories according to the intrarumen pressure, as determined with the tympanometer. Slight bloat was designated for all positive pressures below 20 mm. Hg, and moderate bloat from 20–40 mm. Hg. Bloat was classified as severe when pressures were above 40 mm. Hg and the following symptoms were present: excessive urination and defecation, labored breathing, extreme restlessness, and extention of the right flank as well as the left.

RESULTS

The number of bloat cases and their severity for all experimental periods is shown (Table 1). During each control period, there were 102 possibilities

TABLE 1

Summary of data showing the number of cases of bloat, divided according to the maximum intrarumen pressure in mm. Hg, during the pre-test, test, and post-test alfalfa-grazing periods

Date	No. of cows	Cases of bloat according to the maximum intrarumen pressure *(mm. Hg)*										Total bloat cases	Animals bloated *(%)*
		0-10		10-20		20-30		30-40		>40			
		A.M.	P.M.	A.M.	P.M.	A.M.	P.M.	A.M.	P.M.	A.M.	P.M.		
Break-in period													
9-26	17	0	4	0	1	0	0	0	0	0	0	5	15
9-27	17	0	4	2	4	7	6	1	1	2	0	27	79
9-28	17	1	6	3	7	7	2	4	0	0	0	30	88
Total		1	14	5	12	14	8	5	1	2	0	62	61 (Av.)
Pre-test period													
9-29	17	2	NG	7	NG	5	NG	0	NG	0	NG	14	82
9-30	17	3	NG	1	NG	1	NG	0	NG	0	NG	5	29
10-1[c]	17	3	6	2	4	2	2	0	0	0	0	19	56
10-2	17	1	2	3	4	5	4	6	1	2	0	28	82
Total		9	8	13	8	13	6	6	1	2	0	66	65 (Av.)
Test period[a]													
10-3	17	1	1	0	2	0	0	0	0	0	0	4	12
10-4	16	3	2	0	0	0	0	0	0	0	0	5 (3)[b]	15 (12)[b]
10-5	17	2	4	0	2	0	1	0	0	0	0	9	26
10-6	17	1	NG	0	NG	0	NG	0	NG	0	NG	1	6
Total		7	7 (5)[b]	0	4	0	1	0	0	0	0	19 (17)[b]	18 (16)[b] (Av.)
Post-test period													
10-7	17	1	NG	1	NG	0	NG	0	NG	0	NG	2	12
10-8	17	4	3	3	2	0	1	0	0	0	0	13	38
10-9	17	2	5	5	3	3	1	1	0	1	0	21	62
10-10	17	3	NG	4	NG	4	NG	2	NG	0	NG	13	76
Total		10	8	13	5	7	2	3	0	1	0	49	48 (Av.)

NG — no grazing.

[a] Oat hay was included in the ration during the test period only.

[b] Excludes bloat cases for P.M. grazing on October 4.

[c] Moved cows to less mature half of pasture.

for the development of bloat and, from these possibilities, 66 cases of bloat were observed in the pre-test period and 49 in the post-test period. In the test period, there were 19 cases of bloat out of a total possibility of 117. If the category of 0–10 mm. Hg is eliminated from all periods, the pre-test, test, and post-test periods have the following number of bloat cases, respectively: 49, 5, and 31.

There was an additional afternoon grazing subperiod during the test period. If one of these subperiods (Oct. 4) is arbitrarily eliminated for the purpose of trial uniformity, only 16% of the cows bloated when oat hay was included in the diet. The incidence of bloat was only 4% if the lowest bloat classification (0–10 mm. Hg) is dropped. Under the same conditions, this is compared to 48 and 30%, respectively, for the pre-test and post-test periods.

According to the definitions of slight, moderate, and severe bloat cited above, when oat hay was fed the night before grazing succulent alfalfa, 94% of the bloat cases were slight, with the remaining 6% of the moderate degree. When no oat hay was fed, slight bloat incidence was 58% for the pre-test period and 73% for the post-test period, moderate bloat, 39% and 25%, and severe bloat, 3% and 2%, respectively.

On the basis of four morning and two afternoon grazing subperiods per period, there were 66, 17, and 49 cases of bloat of all degrees during the pre-test, test, and post-test periods, respectively. An analysis of variance indicated that the difference between the number of cases of bloat in the pre-test period and the test period was highly significant. The difference between the number of bloat cases in the test period and post-control period was significant at the 5% level. In other words, the inclusion of oat hay in the diet of cows grazing succulent immature alfalfa reduced the incidence of bloat. Significance was also obtained when the 0–10 mm. Hg degree of bloat was dropped from all trials. Furthermore, an analysis of variance indicated that the presence of oat hay significantly reduced the severity of bloat.

The amount of oat hay consumed at night, previous to the grazing of the alfalfa pasture during the test period, was an average of 12 lb. per cow per night.

DISCUSSION

The results of these experiments confirm the recommendation of Cole and coworkers *(3, 5, 19)*, that an adequate amount of grass hay fed before grazing succulent, immature alfalfa will greatly reduce the incidence and severity of acute bloat.

The inability of oat hay to eliminate the appearance of bloat possibly might be explained on the basis of the amount of oat hay consumed, or that Sudan grass hay is a better bloat preventive than oat hay. If bloat was to be prevented, Cole and Kleiber *(3)* found that it was necessary for the cows to consume at least 17 lb. of Sudan grass hay the night before grazing the bloat-provoking legumes. In the present work, only 12 lb. of oat hay were eaten overnight. Meyer, Lofgreen, and Ittner *(21)* have reported that oat hay feeding eliminated the bloat problem in steers being used to study the utilization of alfalfa pasture. Satisfactory bloat control was obtained also by Hull and coworkers *(13)*, who

prefed 3–5 lb. of Sudan grass hay to steers weighing approximately 650 lb. each. Hogg and Barrentine *(12)* reduced the incidence of bloat in the morning grazing period (but not in the afternoon) by prefeeding 5 lb. of hay. If the prefeeding of hay was permitted on a free-choice basis, more effective control of bloat was achieved. In addition, they commented that hay or grain feeding twice a day might offer effective bloat protection. Recently, Johns *(15)* was unsuccessful in preventing acute legume bloat in dairy cattle by prefeeding 2 lb. of hay. Neither Hogg and Barrentine *(12)* nor Johns *(15)* related the nature of the hay fed. Apparently, the inability of other workers to achieve successful bloat control *(14, 15, 18)* with "hay" rests on the possibility that an adequate amount of a "grass" hay was not fed.

Although they did not measure pasture consumption, Hogg and Barrentine *(12)* and Johns *(15)* expressed the belief that the prefeeding of hay in larger quantities than they used would suppress pasture consumption. On the basis of their alfalfa pasture consumption studies, Cole and Kleiber *(3)* came to the conclusion that, in general, the prefeeding of Sudan grass hay stimulated alfalfa pasture intake. Therefore, it is reasonable to assume that the amount of oat hay ingested in the trials reported here did not limit the intake of alfalfa pasture.

Feedlot and legume bloat are similar, in that both are the result of an intense frothing of the ruminal ingesta *(14, 15, 25, 27)*. In their feedlot bloat studies, Lindahl and coworkers *(18)* reported that a 2:1 ratio of alfalfa hay to concentrates did not relieve the bloating problem in steers. This is not surprising, since Cole, Mead, and Regan *(5)* also earlier found alfalfa hay ineffective in controlling bloat.

In the pre-test period (Table 1), there is a drop in the incidence of morning bloat from 14 cases on September 29 to five on September 30. It will be observed that there was no grazing during the afternoon of the 29th. A similar decrease in morning bloat occurred on October 1, despite the fact that the cows were moved to the less mature half of the pasture. Possibly, this decrease in bloat incidence can be explained on the basis of depressed fermentation caused by a lowered microbial population. The cows had been without feed for approximately 21 hr. and the nutrient intake before this was reduced as a result of bloating. Since there is a periodic movement of contents from the rumen, the microbial population of the rumen constantly is being reduced. In addition, the microbial population might be further decreased because of a lack of available nutrients. Evidence to support these possibilities was reported by Quin *(23)*, who found that the glucose-fermenting ability of sheep rumen fluid dropped dramatically following a 17-hr. fast. By 41 hr. after the beginning of fast, the fermentation was approximately one-third of normal. Quin, Oyaert, and Clark *(24)* tested the cellulose-splitting ability of whole rumen ingesta, strained fluid, and centrifuged fluid during fasting conditions. In the nonfasted state, the cellulose-splitting ability of all fractions was equal. Within 24 hr. after the beginning of fast, the rate of digestion of cellulose in the strained and centrifuged portions dropped sharply to less than half that of feeding. The ability of the whole ingesta to split cellulose did not decrease until 48 hr. after the beginning of fast. Johnson and coworkers *(16)* observed that the bacterial counts in the rumen

were highest 1 hr. after feeding, then declined rapidly thereafter. Similar results were reported by Bortree and coworkers *(1)*, with the exception that the elevated bacterial population following feeding was maintained for several hours. Therefore, on the basis of the foregoing, it might be expected that bloating would be less frequent and certainly less severe in animals fasted for at least 18 hr., than in nonfasted animals, when both groups were subsequently permitted to graze bloat-provoking legumes.

The ability of the alfalfa pasture to produce bloat in the absence of oat hay is shown (Table 1). During the post-test period, there was an expected lag in bloat development. The rumen still contained sufficient amounts of oat hay to prevent all except the mildest degree of bloat for two days. This protection from bloat was augmented somewhat by the lack of afternoon grazing on October 6 and 7.

The mechanism by which grass hays protect ruminants from bloat is not known, but three possible mechanisms have been suggested. Clark and Quin *(2)* proposed that grass hays prevent the formation of a frothy, glutinous mass, presumably by a churning action, and allow the gas to escape from the ingesta. Limited experiments conducted by Ferguson and Terry *(10)* suggest the possibility that grass juice may contain an antifrothing property. Bloat could not be produced consistently in sheep by dosing with 2.5 liters of grass juice containing a foam stabilizer (egg albumin or commercial saponin) and a source of gas (sodium bicarbonate and acetic acid). In one trial, 2.5 liters of a solution containing egg albumin, sodium bicarbonate, and acetic acid, but no grass juice, produced moderate bloat in a sheep. Cole, Mead, and Kleiber *(4)* proposed that scabrous grass hays stimulate the ruminal receptors responsible for exciting the eructation reflex.

Lindahl and coworkers *(18)* found a reduction in the incidence of bloat in a steer when a previously bloat-provoking ration was pelleted. Presumably, only the physical nature of the feed had been changed in the pelleting process. On this basis, these authors comment, ". . . the relationship between the physical nature of the diet and the pathogenesis of bloat cannot be ignored."

Although the length of time the cows were on the alfalfa pasture was too short for the rumen to become atonic, it is quite possible that, during the test period, oat hay may have had a stimulating effect on the rumen motility center.

It is known that cattle on succulent nonscabrous diets ruminate less frequently and chew each bolus for a shorter period of time than when they are fed scabrous diets *(8, 26)*. During rumination, the frequency of rumen contractions is greater than that observed during rest *(8, 11)*; therefore, it might be assumed that the scabrous diet causing rumination also stimulates the rumen motility center. On the other hand, motility is also more frequent during eating than during rest *(8, 11)*, which might involve buccal or pharyngeal receptors. Therefore, the question might be posed: Is the increase in rate of ruminal contraction during rumination the result of the stimulation of rumen receptors or receptors present in the mouth or pharynx? Irrespective of the answer to this question, rumen motility is increased during rumination either directly or indirectly, by scabrous material in the diet.

Many workers have shown *(4, 6, 22, 25, 28, 29, 30)* eructation and rumen motility to be inter-related, i.e., eructation occurs in conjunction with a rumen contraction. It may be controversial as to the origin of the rumen contraction which is associated with eructation *(28, 29, 30)*; however, the fact remains that an eructation takes place at the same time as a rumen contraction. Therefore, if there should be an increase in rumen motility there should be a concomitant increase in eructation rate. That this is the case has been shown by several workers *(6, 9)*.

On the basis of what is known concerning the relation between rumination, rumen motility, and eructation, scabrous material is capable of influencing eructation. It has already been shown that if scabrous material is absent from the diet of cattle for extended periods of time, rumen motility, rumination, and eructation (as evidenced by bloating) are depressed *(7, 20)*.

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DIETARY INFLUENCES ON ERUCTATION AND RELATED RUMINAL PHENOMENA IN CATTLE

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SUMMARY

On a diet of alfalfa tops, eructation rate and volume, as determined by the tracheal transection technique, were so depressed in three steers and two cows that a positive intrarumen pressure was maintained for 130 min. after feeding. Only a slight increase in pressure, which lasted for 70 min., was measured when oat hay was fed the night preceding alfalfa tops ingestion. A subatmospheric intrarumen pressure was found during the oat hay trials.

An eructation occurred on almost every secondary rumen contraction during the oat hay trials. The frequency of the secondary contractions, as well as the total number of rumen contractions, was significantly (1% level) higher during the tops trials than when oat hay was fed. There was no increase in eructation frequency coinciding with the increase in the number of secondary rumen contractions during the alfalfa tops trials. An excellent correlation ($r = 0.96$) was found between the ratio of secondary to primary rumen contractions and intrarumen pressure.

On essentially equivalent TDN intakes, the total gas eructated was greater on the tops than on the oat hay diet.

Frothy rumen ingesta were noted after the completion of the alfalfa tops trials. It is suggested that acute legume bloat results from a rapid rate of gas formation, accompanied by a frothing of the ingesta which physically prevents eructation.

Bloat in ruminants has been divided into chronic and acute *(6)*, with the term acute referring to that bloat occurring as a result of special feeding conditions. Although acute bloat can be produced by using feedlot rations *(23, 35)*, the grazing of legumes is responsible for most of the acute bloat problems *(6)*.

Mead and Goss *(26)* fed Holstein heifers from birth to 18 mo. of age on a ration devoid of roughage. They were normal in size for the breed and showed no abnormalities other than frequent bloating and lack of rumination. The frequency of bloating was attributed to a lack of coarse, irritating material and to ruminal atony, which developed after a considerable period of time *(6)*.

In controlled field studies *(7, 10, 25)* it was found that the appearance of acute bloat resulting from the grazing of soft, succulent legumes could be prevented by including adequate amounts of Sudan Grass hay or of pasture in the diet. It was postulated that the scabrous hay stimulated receptors in the rumen responsible for initiating the eructation reflex.

Studies of eructation were conducted by Weiss *(37)*, who found that he could increase the frequency of the eructation contractions of the rumen of sheep (as determined from a record of ruminal motility) by insufflating the rumen with air. This work would infer that pressure is an effective stimulus for eructation. The contractions of the rumen associated with eructation had been shown previously *(9, 38)*.

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During the course of ruminal motility studies, various workers have observed that contractions of the reticulum and rumen occur in certain ratios *(1, 2, 3, 29, 34)*. Considering contractions of the rumen only, Weiss *(37)* has reported that it was possible to distinguish the following ratios, 1:1, 2:1, 4:1 and, sometimes, 1:2. The ratios refer to the number of "ordinary" ruminal contractions to "eructation" contractions. When the intrarumen pressure was increased by insufflation of air, the number of 1:1 contraction ratios increased.

From the foregoing, it is clear that eructation is intimately associated with ruminal motility and, in fact, takes place at the peak of a rumen contraction *(5, 9, 18, 37, 38)*. When the nerves (vagi) controlling ruminal motility are severed, eructation ceases *(20)*. The events taking place during eructation have been described by Dougherty and Meredith *(16)* and Dougherty and Habel *(15)*.

In an earlier paper, Colvin and coworkers *(12)* described a technique whereby it was possible to determine the frequency and volume of the eructations of cattle. Using this technique, along with the simultaneous recording of ruminal motility, it is the purpose of this paper to show the effect of alfalfa tops, alfalfa tops-oat hay, and oat hay diets on eructation and related rumen activity of cattle. Hereafter, the alfalfa tops and oat hay diets will be referred to as tops and hay, respectively.

EXPERIMENTAL PROCEDURES

The experimental diets considered in this investigation were fresh, immature alfalfa tops (tops), alfalfa tops and oat hay (tops and hay), and oat hay (hay) alone.

The experimental animals were three Holstein steers, each over 18 mo. of age, and two mature Jersey cows. Each animal had a permanent rumen cannula *(12)* or fistula *(9)*, in addition to a tracheal transection described by Colvin and coworkers *(12)*.

The method for collecting and measuring the eructated gas has been described previously *(12)*.

The technique for recording rumen motility and changes in intrarumen pressure was that used at the California Experiment Station for many years, and is illustrated in Figure 1. A similar technique was described by Quin, Van Der Wath, and Myburgh *(33)*.

In general, each trial began with a recording of the fasting rumen motility, followed by the feeding of the experimental ration. During eating, it was impossible to acquire eructation data; however, rumen motility was recorded. Immediately after eating had ceased, the mask and appurtenances of the gas-collection system were installed. In most cases, the duration of the trials was 210 min.

The series of trials conducted on each animal began with the tops ration. This was followed by trials with tops and hay and, finally, with hay alone. In the tops and tops-hay trials, each animal was isolated in a stall without bedding when not being used for measurements.

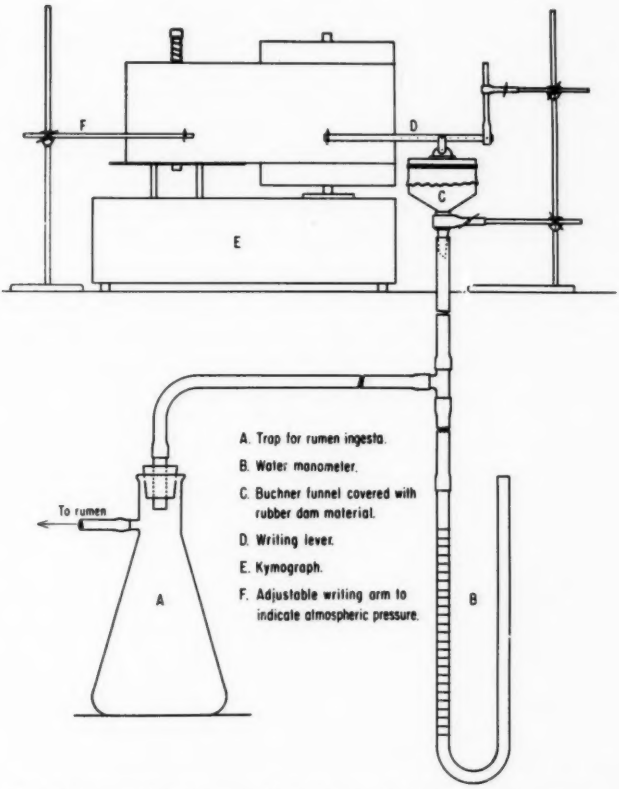

FIG. 1. Apparatus for recording changes in intraruminal pressure.

It was necessary to bring the animals on a full feed of tops very slowly. For some reason, fresh tops are relatively unpalatable when first fed to cattle, although they will consume them eagerly if introduced gradually. Full feed was assumed when the feces became dark green and highly fluid, a condition which usually prevailed within six days of tops feeding. By trial and error, it was found that 10–15 lb. of tops could be consumed in 15–30 min. and would produce experimental bloat. Total individual daily consumption of tops by all animals was approximately 60 lb.

The feeding regime for the tops-hay trials was exactly the same as that explained for the tops trials, with the exception that hay was fed the night before a trial. The hay was fed on a basis of ¾ lb. per 100 lb. of body weight per day.

Oat hay was fed for four days before the beginning of the hay series of trials, to insure that the effects of the hay only were being evaluated. The hay used had been cut at early-head stage, had lots of color, and was medium-to-fine

stemmed. Judging from the manner in which it was eaten, it appeared to be highly palatable. The hay was fed at the rate of 1.5 lb. per 100 lb. of body weight per day.

Water was offered each animal before a trial (between 8 and 9 A.M.), immediately following a trial (between 3 and 4 P.M.), and in the evening (between 8 and 10 P.M.). On a trial day, the animal was fed the experimental ration immediately following the fasting measurements (between 9 and 10 A.M.). On return to the barn, the animal was fed again (between 3 and 4 P.M.). That evening (between 8 and 10 P.M.) the final feeding of the day occurred.

RESULTS

The amount of feed consumed on the various experimental diets is shown (Table 1). The average tops consumption during the tops and tops-hay trials was 14 and 13 lb., respectively. The total daily intake of tops averaged 52 lb.

TABLE 1

Average individual feed consumption on the various experimental diets, including 25 trials with alfalfa tops, 16 trials with alfalfa tops and oat hay, and 19 trials with oat hay

		Alfalfa tops trials		Alfalfa tops–oat hay trials			Oat hay trials	
Animal	Live weight	At trial	Total daily	At trial AT[a]	Total daily AT	Total daily OH[b]	At trial	Total daily
(No.)	*(lb.)*	*Average feed consumption (lb.)*						
802[c]	1350	15	60	15	60	10	5	15
83[c]	800	15	60	12	56	6	4	11
74[c]	800	15	48	15	54	6	3	9
984[d]	950	14	59	13	52	6	3	9
897[d]	950	10	35	9	44	6	3	9
Average	970	14	52	13	53	7	4	11

[a] Alfalfa tops.
[b] Oat hay.
[c] Steer.
[d] Cow.

for the tops trials and 53 lb. for the tops-hay trials. On the average, 4 lb. of hay were consumed during the hay trials, with total daily consumption averaging 11 lb.

The amount of TDN consumed during the trial period on all rations was essentially the same; however, the feeding of a comparable diet of TDN in the form of hay resulted in an increase in the amount of DM on this diet.

Samples of the tops used in the trials were analyzed for total crude protein (method of Association of Official Agricultural Chemists) and were found to contain 34.2% on the average, the range being 30.4–37.6% on a dry weight basis. The succulence and immaturity of the tops are suggested by this high protein content *(22)*.

The average times required to eat the trial allotments of the various diets were as follows: tops, 25 min.; tops of the tops-hay trials, 26 min.; hay, 48 min. The inclusion of hay in the diet did not increase the time spent eating the trial

allotment of the tops of the tops-hay trials. However, when hay became the experimental ration, almost double the time was required to consume the 30% more dry matter.

Since each animal required a longer period of time to consume the trial allotment of hay than it did for the tops, it was necessary to make certain corrections with respect to "time" on the figures cited in this paper. Zero minutes was the time at which the slowest-eating animal began to consume its hay; all other times were then calculated from this. Thus, the consumption of tops (of both the tops and tops-hay trials) began at an average time of 33 min., and hay at 12 min. Tops-eating ended at an average time of 58 and 59 min., for the tops and tops-hay trials, respectively. Hay ingestion ceased at 60 min. Since it required approximately 10 min., on the average, to install the eructation-measuring apparatus, the eructation measurements began at an average time of 70 min. The values for time cited above are averages of five animals, used in the following number of trials: tops, 25, tops-hay, 16, and hay, 19.

Figure 2 illustrates the effect of the various trial diets on the amount of gas eructated at various intervals following feeding. The slope of the tops curve,

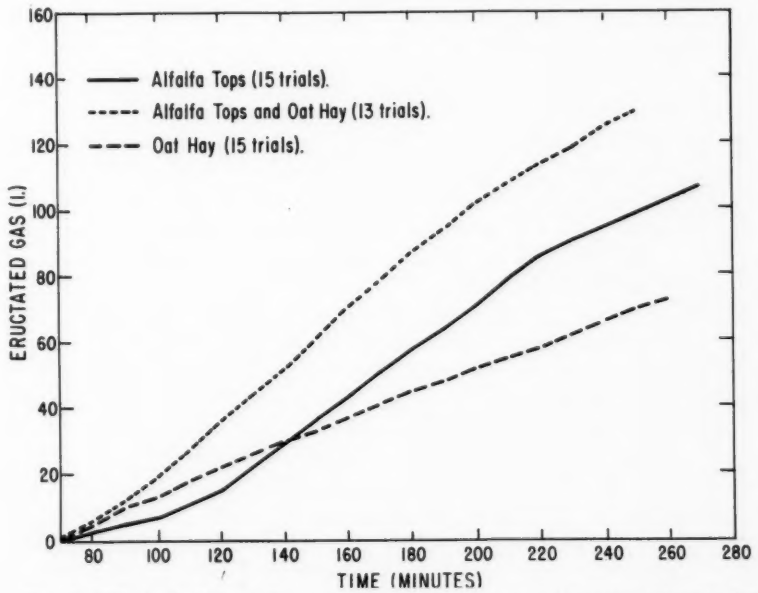

FIG. 2. Effect of diet on the sum of gas eructated at progressive intervals following feeding.

from 70 to 120 min., demonstrates that gas was being retained in the reticulorumen; whereas, no retention was indicated by the tops-hay curve, even though the trial diet also consisted of tops. Note, for example, that at 110 min. an aver-

age of ten liters had been eructated by animals fed tops and 20 liters by animals on hay, even though the total gas released at 250 min. was distinctly higher for the animals on tops. The slopes of the tops and tops-hay curves began to parallel each other at Time 130, indicating that from this point on, the rates of gas eructated were approximately equal.

When the diet consisted of hay alone, the curve of the sum of the gas eructated at progressive intervals was essentially a straight line, indicating that the rumen gas was being eructated as rapidly as it was evolved.

The total amount of gas produced was lowest on the hay diet and highest on the tops-hay diet. An intermediate level of gas production resulted from the tops alone. These differences in gas production probably can be explained on the basis of TDN availability *(21)*. The nutrients of the fresh tops were all readily available to the rumen microflora and, consequently, led to a fulminating gas production. Although the test meal of hay contained as much TDN as the tops test meal, fermentation took place more slowly. The average rates of gas eructated per hour on the tops and hay diets were 32.4 and 22.5 liters, respectively. Under the conditions of these experiments, the amount of gas eructated on the tops and hay diets, respectively, can be estimated from the following equations:

$$Y = 0.54X \text{ and } Y = 0.375X,$$
where Y = the amount of gas eructated in liters
and X = time in minutes.

These equations were calculated from a total of 15 trials per diet, using five animals. The TDN of the test meal of tops of the tops-hay trials was the same as that of the tops trials; thus, it might be expected that comparable amounts of gas would be produced. However, hay was fed each night before a tops-hay trial, which contributed a fermentable residue. Fermentation of this residue undoubtedly was stimulated by the presence of the fresh tops, leading to the evolution of the greatest volume of gas.

According to Figure 3, when the diet consisted of hay, there was a gradual decline in the amount of gas eructated per unit time as the interval of time after eating increased. When the diet consisted of tops alone, the amount of gas initially available for eructation was slight in amount, only a little more than five liters during the first measurement period. The same situation existed following the consumption of the tops of the tops-hay regime, except that it was not as marked. That this gas was trapped in the rumen is indicated by the increase in intrarumen pressure resulting from the consumption of the tops (Figure 4).

The ingestion of tops alone resulted in the greatest intrarumen pressure (Figure 4). Although there was a rapid increase in pressure during the tops-hay trials, it was only one-sixth the magnitude of the tops trials. Atmospheric pressure was never reached when the diet consisted of oat hay.

In Figure 5 is shown the effect of the various diets on eructation frequency. The fewest number of eructations and, thus, the greatest intrarumen pressure, occurred during the tops trials. Even though the greatest number of belches was counted during the tops-hay trials, the frequency was not sufficiently rapid

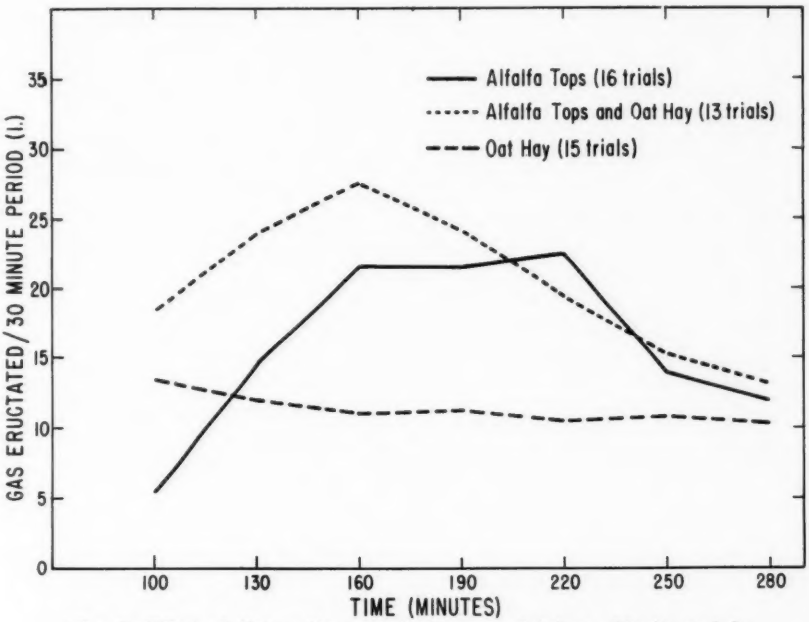

FIG. 3. Effect of diet on the amount of gas eructated per 30-min. period.

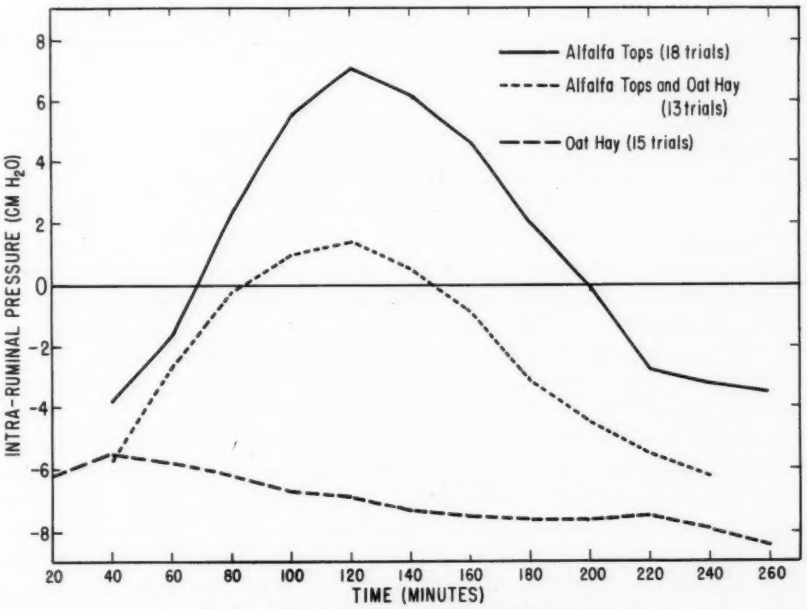

FIG. 4. Effect of diet on intraruminal pressure.

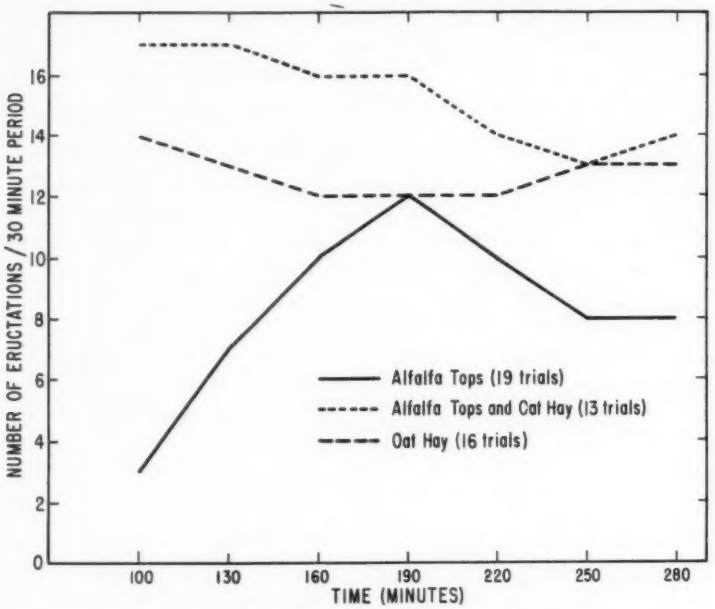

FIG. 5. Effect of diet on the frequency of eructation.

to prevent a slight increase in intrarumen pressure. The frequency of eructation during the oat hay trials was relatively constant, varying between 12 and 14 each 30-min. period.

From the kymograph records of ruminal motility, the number of primary and secondary deflections (contractions) *(18)* were counted and expressed as a ratio of secondary to primary. When rumen motility records are made using the intrarumen pressure technique, the primary deflections are easily differentiated from the secondary, because they are larger. Figure 6 shows that when the diet consisted of hay, the number of primary deflections occurred more frequently than those associated with gas expulsion. When the diet contained tops, there was a marked increase in the number of secondary or eructation deflections, as indicated by an increase in the ratio of secondary deflections to primary. In general, the fasting ratios of contraction appear to be approximately the same, regardless of the diet. There is a direct relationship between the ratio of secondary contractions to primary and the intrarumen pressure ($r = 0.96$). At the maximum intrarumen pressure observed in these studies, the frequency of the eructation or secondary deflections was approximately twice that of the fasting state.

The differences in the maximum eating contraction frequency (Figure 7) between the tops and hay, tops-hay and hay, and tops and tops-hay trials were all statistically significant; the first two at the 1% level and the last at 5%.

Following the ingestion of the hay allotment of the hay trials (Figure 7), the contraction frequency rapidly reverted to the resting state. This was not true following the tops meal of the tops or tops-hay trials, where the contraction

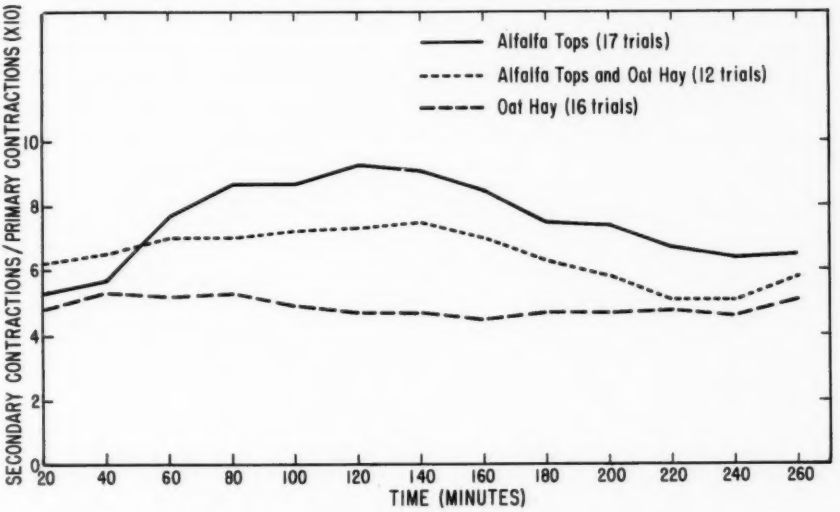

FIG. 6. Effect of diet on the ratio of secondary contractions to primary.

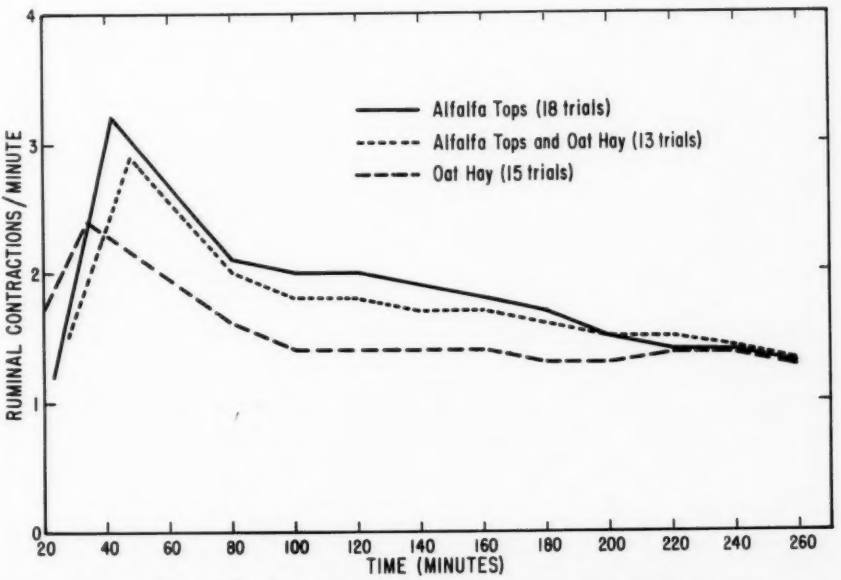

FIG. 7. Effect of diet on the frequency of ruminal contractions.

frequency remained higher than the resting for some time. The increased frequency over fasting corresponds to the time of elevated intrarumen pressure. After the pressure was reduced, the contraction frequency returned to the resting state. Thus, it appears that the number of secondary contractions associated with eructation and the frequency of rumen contractions both increase during elevated intraruminal pressure.

On the average, there were 324 ± 15, 321 ± 10, and 274 ± 6 rumen contractions during the tops, tops-hay, and hay trials, respectively. There was no statistical difference between the values for the tops and tops-hay trials; however, the differences between the number of rumen contractions during the tops and hay and the tops-hay and hay trials were highly significant (1% level).

Of the total number of rumen contractions ,$44 \pm 2\%$, $38 \pm 3\%$, and $31 \pm 3\%$ were of the secondary type during the tops, tops-hay, and hay trials, respectively. The differences between the percentages occurring during the tops and hay trials and tops-hay and hay trials were highly significant (1% level).

The total number of eructations, on the average, during the tops and tops-hay trials were 55 ± 4 and 110 ± 9, respectively. The difference between the two was highly significant (1% level).

DISCUSSION

This investigation was undertaken to obtain basic information concerning eructation in ruminants, especially during the onset of bloat. Cole, Mead, and Kleiber *(9)*, Quin *(31)*, and Wester *(38)* recognized that bloat was in some manner directly associated with the cessation, or at least hindrance, of eructation. The results shown on Figure 3 (gas eructated per 30-min. period) and Figure 5 (number of eructations per 30-min. period) confirm the views of the above workers. Whereas Cole and coworkers *(9)* believed that the effective stimulus for eructation (scratching of the rumen mucosa) was lacking in a diet of young succulent legumes, Quin *(31)* suggested a mechanical blockage of the cardiac orifice with foam. Frothy bloat has been described in an excellent manner by Wester *(38)*.

Little quantitative work done under laboratory conditions exists concerning the bloat problem *(14)*. The inability of researchers to produce bloat at will has been blamed for this lack of basic information *(14)*. At California, we have found it possible to produce rumen distention for laboratory study whenever desired if the animals are fed young, succulent legumes for a sufficient length of time *(10)*.

That the techniques employed in the measurement of the belched gas in no way contributed to the development of bloat is illustrated by the results of the hay trials (Figure 4). With this diet, a positive intrarumen pressure never developed. Certainly, the volume of gas produced would have been sufficient to cause rumen distention had eructation been impeded by the apparatus.

Rumen motility studies have been carried out by numerous workers *(9, 13, 18, 24, 29, 32, 37, 38)*. The cyclical nature of the intrarumen events also has been reported *(1, 29, 37)*. In general, the results of these studies indicate that

there is a ruminal contraction which moves ingesta posteriorly (primary contraction) and another responsible for an anterior displacement of rumen contents (secondary contraction). It has been shown *(37, 38)* that eructation usually takes place on the secondary deflection; hence, the term eructation contraction. That this is undoubtedly so is indicated by the observation that, on the average, there were 92 ± 9 secondary contractions and 88 ± 10 eructations during the oat hay trials. Wester *(38)* comments, "As long as digestion proceeds normally, eructations occur with the regularity of clockwork. In any case, the opening of the cardia always follows the returning contraction wave, regardless of whether audible belches follow or not."

Dougherty and Meredith *(16)* have recently used a cinefluorographic technique to study eructation in a nonbloated sheep. The intraruminal and intraesophageal events revealed by this technique have been described by Dougherty and Habel *(15)*. According to the latter workers, after the gas has been moved to the region of the cardia by rumen contraction and dilation of the reticulum, the cardiac and diaphragmatic sphincters open. The rumen gas then has a free passageway up to the cranial esophageal sphincter. The two caudal sphincters close, to be followed immediately by the opening of the cranial esophageal sphincter. The gas is forced from the esophagus out into the atmosphere as a result of the contraction of the esophageal musculature and increased intrathoracic pressure.

As only a small volume of gas is belched per eructation during the nonbloated condition (0.5 to 1.7 liters), it is conceivable that the esophagus offers ample space to receive the gas released from the rumen during one belch. However, it seems rather doubtful that the esophagus of the bovine is capacious enough to accommodate a volume of 4–6 liters at one time. There were occasions when the volume of gas per eructation exceeded the upper level indicated. During these extenuating circumstances, it seems reasonable that the cephalic esophageal sphincter might open at the same time as the lower sphincters, to facilitate eructation when large volumes of gas are to be voided. This may very well be one of the compensating mechanisms brought into play by the animal as a result of excessive intrarumen pressure. Wester *(38)* comments on another possible compensating mechanism as an aid in the reduction of increased intrarumen pressure. He says that, under nonbloated conditions, the cardiac orifice opens completely in conjunction with the forward-moving contraction wave. When the animal is bloated, the orifice may open along with the posterior-moving contraction. We have observed events similar to those described by Wester *(38)*, i.e., eructations taking place in conjunction with the primary deflections as well as with the secondary deflections. Parsons and coworkers *(28)* observed several belches which took place during the relaxing phase of primary rumen contractions.

Wester *(38)*, Cole and coworkers *(6)*, and the present authors have observed eructation occurring in an animal with an open rumen fistula. The existence of a cranial esophageal sphincter *(15)* may offer an explanation for these observations. Before gas can pass from one region to another, there must be

either a pressure or a concentration gradient, or an active transporting mechanism. In the case of eructation in the ruminant, only the pressure gradient need be considered.

In the intact animal, or in the animal having a closed rumen fistula, eructation usually takes place at the peak of a rumen contraction wave *(9, 32, 37, 38)*, i.e., the forward-moving contraction wave. At this time, the pressure in the rumen is elevated, so that when the cardiac orifice opens the gas rushes into the esophagus. In quiet breathing, the intrathoracic and, thus, intraesophageal pressure is subatmospheric, especially at the time of inspiration. This relatively negative pressure facilitates the entrance of the gas into the esophagus from the rumen.

In the fistulated animal whose fistula is left open to the atmosphere, the subatmospheric pressure in the esophagus may be solely responsible for the passage of gas from the rumen (and thus the atmosphere) into the esophagus. At the time of the eructation contraction, the cardiac orifice would be opened as usual. Since the intraesophageal pressure would be subatmospheric, gas would enter the esophagus up to the level of the cranial esophageal sphincter. Shortly thereafter, the cardiac sphincter would close and the cranial sphincter open. The increase in intraesophageal pressure resulting from a contraction of the esophageal musculature and an expiratory effort would force the esophageal gas out into the atmosphere.

The number of papers concerned with the measurement of gas evolved by fermentative processes in the rumen is extremely limited *(8, 9, 30, 36)*. Immediately following the ingestion of fresh legumes, there is a sudden onset of activity by the rumen microflora which results in a rapid gas evolution *(8, 9, 30, 38)*. Hungate and coworkers *(21)* conducted in vitro studies with Ladino clover juice and rumen ingesta. They found that an "explosive fermentation" resulted. When macerated Ladino clover was placed in the fermentation flasks in place of the juice, rapid fermentation followed, but not at the rate observed with the juice. The difference in rate of fermentation was attributed to differences in availability of the fermentable substances. The present in vivo work substantiates these findings.

Cole and coworkers *(9)* and Quin *(31)* insufflated gas into the rumen of animals at a rate far exceeding normal gas production by rumen microorganisms. They found that the gas was belched away with ease. After observing this, Cole, Mead, and Kleiber *(9)* stated that excessive gas formation was not the main cause of bloat.

Although there is little doubt that "excessive" gas formation is not the main cause of acute ruminant bloat, it is becoming increasingly apparent that "rapid" gas evolution may be an important etiological factor. Intraruminal pressures were greatest (Figure 4) on the diets which resulted in the most rapid rate of gas production (Figure 2). When coupled with a decreased rate of eructation (Figure 5) and volume of gas per eructation (Figure 3), the rate of gas production becomes exceedingly important.

The increase in intrarumen pressure (Figure 4) following the consumption of the tops, when the experimental diet consisted of tops alone, resulted from two different factors: *(a)* a low volume of gas eructated per unit time (Figure 3) and *(b)* a low frequency of eructation per unit time (Figure 5). When hay was added to the tops diet, there was still an increase in intrarumen pressure over that normally present in the rumen as indicated by the hay curve. Since the frequency of eructation was high during the tops-hay trials (Figure 5), the increase in intrarumen pressure must be explained by a decreased amount of gas per eructation (as shown by Figure 3). It is of interest to note the speed with which a positive intrarumen pressure develops.

During the tops-hay trials, there was retention of enough gas in the rumen to produce a pressure greater than normal. However, the pressure developed more slowly (Figure 4), reached only one-sixth the magnitude, and returned to resting sooner than comparable measurements made during the tops trials. Furthermore, the eructation rate (Figure 5) during the tops-hay trials was the highest of all the experimental rations. In field trials, 4–7 lb. of Sudan Grass hay did not prevent bloat in mature cows when fed 2 hr. preceding the grazing of immature alfalfa pasture *(7)*. On the other hand, Meyer and coworkers *(27)* found that 4–7 lb. of oat hay prevented bloat in 480-lb. steers on immature alfalfa pasture.

It has been claimed, without supporting evidence, that the frequency of rumen contractions increased during the onset of bloat *(6, 37, 38)*. That the foregoing appears to be the case, is indicated by the increased frequency of rumen contractions during the period of elevated intrarumen pressure (Figure 7).

The data show that no increase in eructation frequency coinciding with the increase in frequency of the eructation contractions occurred during the tops trials. The evidence indicates that the elevated intrarumen pressure might have been responsible for the increase in the number of eructation contractions. This is shown by the direct relationship ($r = 0.96$) of the ratio of the secondary contractions and primary contractions to the development of intrarumen pressure. In his rumen insufflation studies on sheep, Weiss *(37)* has demonstrated that an increase in intrarumen pressure is accompanied by an increase in the frequency of eructation contractions. Thus, in the animal suffering from legume bloat, there appears to be an initial unsuccessful reflex attempt to increase the number of eructations by increasing the number of eructation contractions.

Following the completion of the tops trials, it was observed that the rumen ingesta were extremely frothy in nature. It became apparent that the froth formed during the rapid fermentation of the succulent legumes was either blocking or partially blocking the cardiac orifice and preventing eructation. Other experiments with antifrothing agents add credence to this possibility *(11)*.

If the assumption is made that frothing of the rumen ingesta is an important etiological factor in acute legume bloat, how can the effect of grass hay in bloat prevention be explained? Is it possible that the hay in some manner, perhaps by churning *(4)* or by direct antifoaming action (a chemical factor), prevents the accumulation of large amounts of foam? If this is the case, the nature of the

effective stimulus (scratching or pressure) for increasing eructation under the conditions of legume bloat becomes of less importance than previously supposed. The first consideration for bloat treatment or prevention would be foam dispersion. Following this, or perhaps simultaneous with it, the importance of stimulating or of reinforcing the eructation reflex would become paramount. That the frequency of the secondary or eructation contractions should have been adequate to reduce the intraruminal pressure resulting from the rapid fermentation of the tops, is indicated (in Figure 6). Apparently, the frothing was sufficiently intense to occlude the cardia and to render the eructation reflex ineffective. When no froth was present, as was the case in the hay trials, an eructation occurred on almost every secondary contraction.

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SOME OBSERVATIONS ON THE USE OF SODIUM PROPIONATE FOR THE PREVENTION OF KETOSIS IN DAIRY CATTLE [1]

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SUMMARY

The herd of Guernsey cattle owned by the University of Delaware, which had previously shown a very high incidence of ketosis, was divided into control and treatment groups which were as near alike as possible in history of ketosis. Cows which calved a second time during the course of the experiment were placed in the group opposite their original group. In this way, 11 of the cows were included in both groups. The treatment group received one-eighth of a pound of sodium propionate twice daily by drench for approximately 6 wk. postpartum. The effect of propionate upon incidence of ketosis, blood sugar levels, blood ketone levels, and milk production was investigated. The incidence of ketosis was low in both groups, but it was just as high in the treated as in the control. There was some tendency for the treated group to be lower in blood ketones and higher in blood glucose than the control group; however, individual variation was too great for these differences to be significant. There was a highly significant difference in milk production favoring the treatment with sodium propionate.

Utilization of the glucogenic properties of propionic acid in the treatment and/or prevention of ketosis in dairy cattle has been reported by Schultz *(4, 5)*. He has reported higher blood sugar, lowered blood ketones, and higher milk production when cows in ketotic herds were fed sodium propionate. Schmidt and Schultz *(3)* reported no differences when ten "normal" cows were used in a double-reversal type of experimental design.

The following is a report of the use of sodium propionate in the herd of Guernsey cattle at the University of Delaware.

EXPERIMENTAL PROCEDURE

In the two years prior to the initiation of this study, the herd had experienced a high incidence of ketosis (about 75%). All cows in the herd were divided into two similar groups, based on their ketotic history. One group received one-eighth of a pound of sodium propionate twice daily by drench for the first 6 wk. postpartum. The other group received no treatment unless symptoms of ketosis were observed. There were 17 cows in each group, the average ages of which were 5.9 and 6.1 yr. for the treated and control groups, respectively. The study continued 21 mo. Cows that freshened a second time in this experimental period were placed in the opposite treatment group. This was done so that, insofar as possible, each cow could serve as its own control. There are 11 cows which were thus included in both groups; the average ages of these were 5.5 and 5.6 yr., respectively.

Blood samples were taken weekly at approximately 9 A.M. and analyzed for

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[1] Delaware Agricultural Experiment Station Miscellaneous Publication 306.

glucose by the Nelson modification *(2)* of the Somogyi-Shaffer-Hartman method *(6)*, and ketones were determined by the method of Behre *(1)*. Sodium oxalate was used as the anticoagulant. The average daily milk production for each week was calculated from the milk weights recorded at the barn.

RESULTS AND DISCUSSION

The incidence of ketosis was much lower than in the previous year. One cow (No. 4) in the treated group developed all of the classical symptoms of ketosis at 12 days postpartum—blood glucose 23 mg. %, ketones 38 mg. %, poor appetite, and some reduction in milk production. It failed to respond to treatments given, including ACTH, ACK Vet tablets[2] (mainly aspirin), and intravenous glucose. It recovered at 6 wk. postpartum after ten days of a final treatment with 250 ml. per day of 50% dextrose intravenously. This may have been a spontaneous remission.

Two others (No. 12 and No. 22) in the treatment group developed what appeared to be the nervous type of ketosis; however, both blood sugar and ketones were normal when nervous symptoms were first noted (Cow 12: 60 and 6.7 mg. %, and Cow 22: 74 and 3.2 mg. %). Both cows recovered promptly upon treatment with a small dose of ACTH plus aspirin (ACK Vet tablets).

In the control group, No. 19 was showing lack of appetite, relatively low blood sugar (41 mg. %), and moderately high blood ketones (14.8 mg. %). The use of one-half pound of sodium propionate daily was effective in preventing ketosis; one-fourth pound was not (Figure 1). No. 9 developed ketosis immediately prior to the planned activation of the project, so is not actually a part of either preventive treatment group. Sodium propionate was used therapeutically, but only at one-fourth of a pound daily (Figure 1). The blood picture was promptly corrected, but the appetite remained extremely poor.

The effect of sodium propionate upon blood glucose, blood ketones, and milk production during the first 6 wk. postpartum in apparently normal cows was investigated as follows: Only cows which showed no demonstrable loss in appetite and/or reduction in milk production were included in Table 1 (13 treated and 14 control cows). The average ages of these cows were 5.93 and 6.08 yr. for the treated and control groups, respectively. There were eight cows that were in the experiment for two lactations, one as a control and another as a treated animal. Of these, three were first in the treated group and secondly in the control group; whereas, the other five were first control and then experimental. Statistically, there was a highly significant difference in milk production favoring the treated group for the entire experiment, but not for the production during any particular week. The average ages are near enough alike to eliminate any marked age bias. The differences in blood ketones and blood glucose levels were not significant.

The eight cows mentioned above were also considered as a separate group

[2] Acetylsalicylic acid, menadione, and ascorbic acid (G. F. Harvey Co., Saratoga Springs, N. Y.).

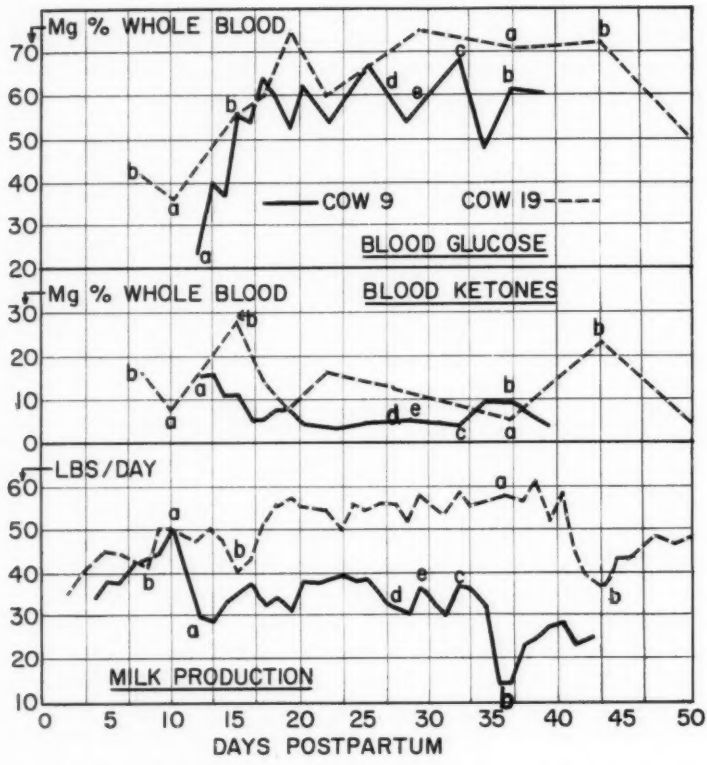

FIG. 1. The effect of sodium propionate upon the blood glucose, blood ketones, and milk production of two ketotic Guernsey cows.

[a] Started ¼ lb. propionate. [b] Started ½ lb. propionate. [c] Discontinued propionate. [d] 1,000,000 units of penicillin. [e] 250 ml. of 50% dextrose.

(Table 2), so that a more complete statistical analysis of milk production could be made (Table 3). With each cow appearing in both treatment groups, cow differences and interactions could be considered. The average ages of these eight cows were 5.9 and 5.7 for the treated and control groups, respectively. Five of these were mature cows (5.5–7.9 yr.) when first placed on experiment. Of the three younger cows, one started in the treated group at 3 yr., 5 mo. of age, whereas the others started as control at ages 2 yr., 5 mo., and 3 yr., 5 mo. With five mature cows and three young ones in this analysis, distributed as above, it is unlikely that the differences in age could account for the milk production differences observed.

There were several missing values in blood ketone levels, so a detailed statistical analysis was possible for only six of the eight cows considered above. The ketone levels are shown (Table 2). None of the differences was statistically sig-

TABLE 1

Blood ketones, blood glucose, and milk production of Guernsey cows
Average of all cows not showing clinical ketosis

Weeks	Blood ketones *(mg. % whole blood)*		Blood glucose *(mg. % whole blood)*		Milk production average/day *(lb.)*	
	Treated[a]	Control	Treated	Control	Treated	Control
1	5.88 ± 1.08[b]	6.12 ± 1.62	45.3 ± 3.12	47.1 ± 3.50	36.7 ± 1.46	34.9 ± 1.72
2	7.00 ± 1.48	7.95 ± 1.40	44.8 ± 2.69	42.8 ± 3.05	41.4 ± 1.75	37.9 ± 1.47
3	6.74 ± 1.30	7.61 ± 1.77	55.0 ± 6.24	49.4 ± 4.16	43.8 ± 1.68	40.5 ± 1.99
4	7.07 ± 1.77	7.35 ± 1.99	53.6 ± 6.30	55.4 ± 4.54	46.0 ± 1.65	42.4 ± 2.23
5	4.48 ± 0.47	5.25 ± 0.88	59.8 ± 6.10	55.6 ± 3.37	46.2 ± 1.87	43.3 ± 2.23
6	5.68 ± 1.29	4.12 ± 0.60	58.0 ± 6.46	60.5 ± 5.14	46.2 ± 1.90	43.1 ± 2.04
Average of 6 wk.	6.17 ± 0.52	6.69 ± 0.66	52.6 ± 2.20	50.9 ± 1.70	43.4 ± 0.79[c]	40.3 ± 0.85

[a] Drenched with one-eighth of a pound of sodium propionate twice daily for 6 wk. postpartum.
[b] Standard error of the mean.
[c] The difference between these means is highly significant.

TABLE 2

Average blood ketones and milk production when each cow was in both the treatment and control groups

Weeks	Blood ketones (6 cows) *(mg. % whole blood)*		Milk production average/day (8 cows) *(lb.)*	
	Treated[a]	Control	Treated	Control
1	5.12 ± 1.36[b]	3.90 ± 0.69	37.0 ± 2.02[c]	34.2 ± 2.66
2	7.70 ± 2.37	7.15 ± 2.65	41.4 ± 1.73[c,d]	37.7 ± 2.57
3	7.23 ± 2.66	8.83 ± 3.48	42.8 ± 1.60[c]	40.6 ± 2.99
4	6.02 ± 1.97	9.82 ± 4.10	45.1 ± 2.11[c,d]	41.4 ± 3.45
5	4.82 ± 0.83	5.72 ± 1.48	45.2 ± 2.59[c]	42.6 ± 3.43
6			45.0 ± 2.53[c]	42.9 ± 3.15
Average	6.18 ± 0.84	7.08 ± 1.19	42.7 ± 0.94[c]	39.9 ± 1.26

[a] Drenched with one-eighth of a pound of sodium propionate twice daily for 6 wk. postpartum.

[b] Standard error of the mean.

[c] The difference between these means is significant at the 1% level when interactions are removed.

[d] The difference between these means is significant at the 5% level when interactions are included in the error term.

[e] The difference between these means is highly significant, using the error term with or without the interactions.

TABLE 3

Analysis of variance of the milk production of eight cows, each of which appeared in both treatment groups

Source of variation	Degrees of freedom	Sum of squares	Mean square	F ratio (Error A)	F ratio (Error B)
Total	95	5,768.30	60.719		
Treatments	1	190.41	190.41	86.95[a]	16.93[a]
Cows	7	3,804.02	543.43	248.14[a]	48.30[a]
Weeks	5	851.32	170.26	77.74[a]	15.13[a]
Interactions					
Week × treatment	5	9.70	1.94	0.99	
Week × cow	35	266.27	7.61	3.47[a]	
Treatment × cow	7	539.05	77.01	35.16[a]	
Error A	49	107.53	2.19	s = 1.479	
Error B (omitting interactions)	82	922.55	11.25	s = 3.35	

[a] Significant at the 1% level.

nificant. A similar situation of missing values made a detailed analysis of blood glucose levels seem impractical.

Apparently, the inclusion of one-fourth of a pound of sodium propionate in the ration of cows in this herd for the first 6 week. postpartum affected the nutrient metabolism such that more milk was produced than when cows were not so treated. This substantiates the work of Schultz *(5)*. The data collected on blood ketone levels and blood glucose levels show some trends toward lower ketones and higher glucose when treated with propionate, but there were very large individual variations and the number of observations in this experiment is too few to show significance.

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PRODUCTION OF GAS IN RUMEN FERMENTATIONS AND STABLE MASS FORMATION FROM ALFALFA FIBER AND PECTIC SUBSTANCES

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SUMMARY

A series of laboratory experiments was carried out to help identify some of the chemical characteristics of alfalfa that were considered a part of the complex causing stable foam formation in the rumens of cattle. Gas production in in vitro rumen fermentations, and stable foam formation after mechanically blending with water, were determined, using various fractions of alfalfa and other substances. The results show that the plant substances responsible for the initial rapid gas production obtained with green alfalfa were closely associated with the fiber fraction, but were removed after digesting in vitro 4–10 hr. with rumen microorganisms or by extracting 12 hr. with hot water. Purified pectin, crude pectins, glucose, and galactose increased gas production when added to washed alfalfa fiber. Hydrolyzed pectin produced the most marked effect, yielding 2.25 times as much gas as the washed fiber. Concurrent hydrolysis of macerated alfalfa with pectinase during digestion also increased gas production.

Added pectin decreased the amount of plant material required to form a stable mass and increased the stable ingesta volume when incubated with rumen contents in vitro.

The experimental results indicated that the combined effects of the physical structure of green alfalfa fiber, pectic substances of alfalfa plants, galacturonic acid obtained on hydrolysis of pectic substances, and reducing sugars normally present are capable of causing the formation of stable foam found in pasture bloat.

There are numerous reasons why excessive quantities of gas are retained in the rumen of cattle. The most prevalent clinical manifestation of bloat in ruminants occurs on legume pasture and results from the formation of a stable mass *(8, 12, 14, 18)*. Pasture bloat has been produced experimentally with various legumes *(2, 6, 8)*, including red clover stems *(11)*. A part of the stable foam is the result of excessive amounts of gas produced when legumes are exposed to rumen fermentation *(3, 16)*. This gas production may arise from decarboxylation of amino acids and organic acids *(5, 17)* or the dissimilation of free sugars *(5, 16)*.

Pounden *et al. (14)* have used in vitro laboratory techniques in attempts to isolate the physical factors of green alfalfa and Ladino clover which cause them to form a stable, foamy mass in the rumen. They have shown that the fiber fraction of fresh alfalfa and Ladino clover, produced by cold-water extraction in a Waring blendor, promoted a more stable type foam than fiber from bird's-foot trefoil and some of the grasses. It also was noted that the fiber-free water extract of legumes and grasses formed about the same amount of nonstable foam. These results were interpreted to indicate that green alfalfa and Ladino clover cell wall structures, froth-stabilizing agents, and rapid gas production combine to form a stable foam in the rumen.

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More recent experiments showed that alfalfa fiber extracted with cold water also contained substances, heretofore not considered, that are excellent precursors of rapid gas production when incubated with rumen microorganisms in vitro. In this study, amounts of gas produced by rumen microorganisms acting on various fractions of alfalfa in vitro were determined. Other products tested were various pectins, glucose, and galactose. These experiments and results are described below. Results of preliminary experiments designed to identify the substances involved also are given.

EXPERIMENTAL PROCEDURES

Rumen contents for inoculating flasks were obtained by stomach tube at 1 P.M. on alternate days from two dairy calves weighing 400–500 lb. Feeding procedures for the calves were standardized, to avoid as much as possible daily variations in the inoculum. Each calf was fed 1 lb. of grain and approximately 3 lb. of mixed hay in the morning, and 1.5 lb. of grain plus all the hay they would eat in the evening. Total hay consumption was 12–16 lb. per calf per day.

The following procedure was used for the short time in vitro fermentations: Fresh rumen contents were strained through a double thickness of 90-gauge cheese cloth. Thirty-five milliliters of strained rumen juice were added to duplicate fermentation flasks to which had been added previously 35 ml. of 1/15 *N* phosphate buffer solution (buffered at pH 6.8) and the substrates to be tested. A temperature of 38.5° C. was maintained throughout the fermentations, using a constant-temperature water bath. Fermentation time was 2 hr. The amount of gas produced was measured as the volume displaced in water manometers attached to each fermentation flask in a closed system, the base of the water column being in equilibrium with the atmosphere. Gas production was measured at 10-min. intervals for either a 1- or a 2-hr. period.

The following procedures were used for preparing substrate materials tested for total gas-producing capacity in short-time in vitro fermentations. Newly cut plant material was macerated in a Hobart food cutter. The washed alfalfa fiber was obtained by repeatedly extracting green alfalfa with cold water in a Waring blendor until all visible traces of green material were removed. The digested washed fiber was obtained by digesting with rumen microorganisms for 2–10 hr. in the fermentation flasks and rewashing with cold water. Alfalfa fiber and alfalfa juice were also separated by expressing the juice from macerated plant material with 15,000 p.s.i. of pressure in a Carter press.

Depectinated alfalfa was obtained by extracting macerated alfalfa with distilled water at 80° C. for 12 hr. *(13)*. Both the extract and depectinated residue were used for rumen fermentations.

A hydrolysate of methyl ester of polygalacturonic acid was obtained by incubating purified pectin at 37° C. for 24 hr. with one-tenth part of 5% pectinase solution, buffered at pH 6.8.

Alfalfa cellulose was prepared from the green plant by treatment with the Crampton and Maynard *(4)* analytical procedure.

Total pectic substances were determined as the alcohol precipitate *(13)*.

A stable foam test *(14)* was used to measure the ability of alfalfa plant material to simulate the stable foam in the rumen contents. Green alfalfa plants were clipped into sections 1 to 2 in. in length, added in successive 2- to 3-g. quantities to 100 ml. of water in an operating Waring blendor, until a stable gassy mass was produced. Stable ingesta volume increase was determined by the method of Jacobson *et al. (7)*.

RESULTS AND DISCUSSION

It was found that washed alfalfa fiber produced an average of three times the amount of gas obtained with an equal weight of alfalfa juice. These data are shown (Table 1). The amount of juice which could be expressed from alfalfa plants by 15,000 p.s.i. of pressure varied as widely as 43 to 85% of the wet weight on successive days. When the juice and fiber from 5 g. of alfalfa were separated in this manner, the amount of gas produced in in vitro fermentations was greater for the fiber fraction, but the total of the two fractions was the same as the original material (Figure 1). When the juice and fiber were remixed together and digested, the amount of gas produced exceeded the amount produced by the original material (Figure 1).

TABLE 1

Effect of adding various fractions of green alfalfa plants on the amount of gas produced in in vitro rumen fermentation [a]

Fraction of alfalfa plant	No. of tests	Amount added	Amount of gas produced
		(g.)	*(ml/hr)*
Green alfalfa [b]	10	5	8.3 ± 1.9
Alfalfa juice	5	5	1.8 ± 1.4
Alfalfa fiber [c]	9	5	5.3 ± 0.15
Digested alfalfa fiber [d]			
(1) Digested 2 hr.	6	5	3.7 ± 0.79
(2) Digested 4–10 hr.	4	5	0.6 .....
Alfalfa cellulose	4	5	1.1 ± 0.21
Hot-water-extracted green alfalfa [e]	3	From 5	1.3 ± 0.54
Hot-water extract of green alfalfa [e]	5	From 5	2.3 ± 0.41

[a] Fermentations consisted of 35 ml. of 1/15 molar phosphate buffer, 35 ml. of rumen juice, and the added alfalfa material.

[b] Green alfalfa macerated in a Hobart food cutter.

[c] Green alfalfa washed with cold water in a Waring blendor.

[d] Digested with rumen microorganisms for the indicated amount of time.

[e] Extracted in water bath at 80° C. for 12 hr.

These results indicated that plant substances responsible for the initial rapid gas production obtained with green alfalfa were closely associated with the fiber. The results (Table 1) also show that the gas-producing substances can be removed after digesting 4–10 hr. with rumen microorganisms or by extracting with hot water. Cellulose determinations of the digested and undigested washed fiber revealed that digested fiber contained a higher percentage of cellulose than undigested fiber, showing that materials other than cellulose were the principal precursors of the gas produced.

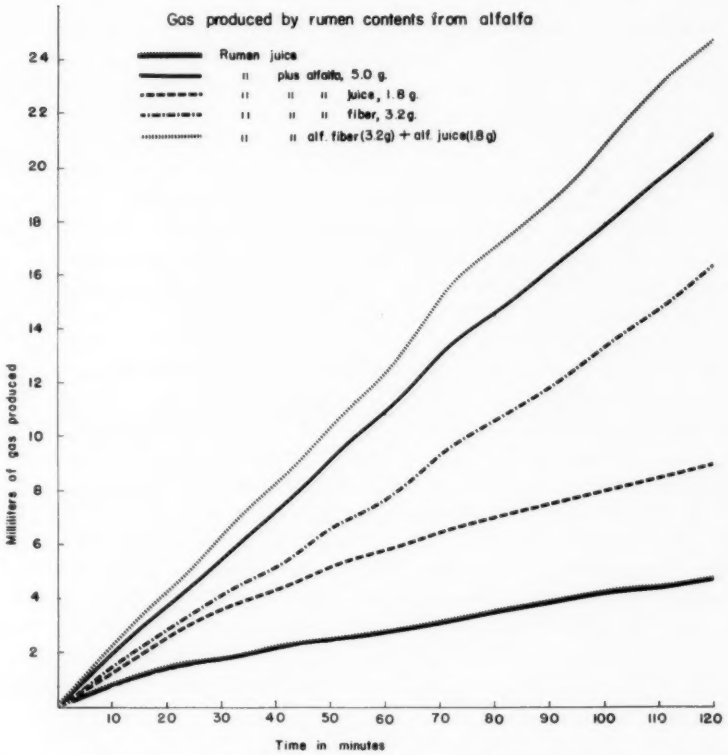

FIG. 1. The total gas produced in vitro by the action of rumen microorganisms on alfalfa juice, alfalfa fiber, and whole alfalfa.

Adding various carbohydrate compounds to washed alfalfa fiber enhanced gas production (Table 2). Purified pectin, crude pectins, glucose, and galactose all increased gas production somewhat. However, 0.3 g. of hydrolyzed pectin produced the most marked effect, yielding 2.25 times as much gas as 2 g. of washed fiber alone. Gas production of macerated alfalfa was increased by adding pectinase to the fermentation mixture. This suggests that hydrolysis of pectic substances may have played an important role in the increased gas production from alfalfa. Analyses showed that alfalfa plants contained 5–8% pectic substances.

A test of the effect of pectin on stability of the mass produced using the mass test showed that on the average 52 g. of green alfalfa produced a stable foam in 8 min. of blending time. When 0.5 g. of crude pectin was added, a stable foam was produced with only 43 g. of plant material in 7 min. of blending time. Using 1 g. of crude pectin, 40 g. of plant material produced a stable foam in 5 min. of blending time.

TABLE 2

Gas produced in in vitro rumen fermentations[a] from adding various carbohydrate compounds with and without washed alfalfa fiber

Substrates used	Amount of carbohydrate added	Amount of gas produced
	(g.)	*(ml/hr)*
Rumen juice	0.0	1.8
Washed fiber plus rumen juice	0.0	5.6
Purified pectin plus rumen juice	0.3	4.1
Purified pectin plus washed fiber plus rumen juice	0.3	7.2
Quickset pectin plus washed fiber plus rumen juice	0.3	9.4
Slowset pectin plus washed fiber plus rumen juice	0.3	9.9
Hydrolyzed pectin plus rumen juice	0.3	8.5
Hydrolyzed pectin[b] plus washed fiber plus rumen juice	0.3	13.5
Glucose plus washed fiber plus rumen juice	0.3	9.9
Galactose plus washed fiber plus rumen juice	0.3	9.0

[a] Fermentations consisted of 35 ml. of 1/15 molar phosphate buffer, 35 ml. of strained rumen fluid, 2 g. of washed alfalfa fiber.

[b] Methyl ester of polygalacturonic acid hydrolyzed with pectinase in buffer solution. (Pectin N.F., courtesy Products Department, Sunkist Growers, Ontario, California, U.S.A.)

Applying procedures for measuring stable ingesta volume increase in vitro *(7)*, it was found that the stable ingesta volume of 100 ml. of untreated rumen contents increased 27 ml. in 45 min. When 3 g. of pectin were added to this amount of rumen contents, stable ingesta volume increased 56 ml. Also, the liquid layer below the stable foamy mass was only 26 ml. when pectin was added, in contrast to 58 ml. of liquid layer in the untreated rumen contents. Thus, the total stable foamy mass was 69 ml. in 100 ml. of untreated rumen contents after 45 min. of fermentation, and 130 ml. when 3 g. of pectin were added.

DISCUSSION

The series of laboratory experiments presented have shown that the plant substances responsible for the initial rapid gas production obtained with green alfalfa were closely associated with the fiber fraction, but were removed after digesting for a short time with rumen microorganisms or extracting with hot water. The magnitude of the increased gas production above that produced by washed fiber was similar to the amount produced using the simple sugars, glucose and galactose. The pectic substances and hemicelluloses primarily may have been involved, since they are hydrolyzed readily to their component sugars and galacturonic acid. Also, it was shown that pectin increased gas production. Hydrolyzed pectin produced the most marked effect, yielding 2.25 times as much gas as washed fiber. It is concluded that the cell wall structures, hemicellulose, and pectic compounds are precursors for rapid ruminal gas formation. These compounds which have not previously been considered in the etiology of bloat may form more than 50% of some cell wall structures.

Among the plant constituents, saponins *(8, 10)*, reducing sugars *(16)*, and albuminous proteins *(1, 6, 8)* have been suggested as singular causes of legume bloat. However, the experimental results in this and other reports *(14, 15)* indicate that excessive quantities of stable foam may arise from the interaction

of several units of the legume plant cell: that is, the cell wall, the middle lamella, and the cell sap. In justification of this theory it was shown that green alfalfa fiber composed principally of cell wall structures provided the structural network for a stable foam when mechanically blended with water or rumen liquid *(14)*. The fiber of bird's-foot trefoil and grasses did not hold liquids in a stable mass.

The middle lamella contains most of the pectic substances as hydrophilic colloids. The experimental evidence presented indicates that they are involved in the formation of stable masses with alfalfa. This was particularly suggested by the indirect evidence, which showed that crude pectin added to either rumen contents or mechanically blended alfalfa with water decreased the amount of plant material required to produce a stable mass. Other points to be noted are: (1) Pectins are present in significant quantities, representing 5–8% of the dry plant weight. (2) The viscosity of methoxylated pectic substances is maximum in the pH range 6.0–6.3 *(9)*. (3) The presence of minute quantities of polyvalent ions such as calcium increases viscosity *(9)*. (4) Partial hydrolysis of proto-pectins increases hydration so that some proto-pectins may hold a thousand times their own weight in water *(9)*. (5) The pectic substances provide rigidity for cell protoplasm during periods of rapid growth and are later displaced. This periodicity of presence of pectic substances is an interesting consideration, since incidence data on the occurrence of bloat indicated that severe bloating periods in this area extended from one to four days on growthy pastures *(15)*. (6) Pectin yields 70–80% galacturonic acid upon hydrolysis and will hydrolyze readily *(9)*. Galacturonic acid was shown to be an excellent precursor to ruminal gas formation in these experiments.

It appears that the combined effects of the physical structure of green alfalfa fiber, its pectic substances, and galacturonic acid produced by hydrolysis of pectic substances are capable of causing the formation of the stable foam found in pasture bloat.

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INFLUENCE OF PURIFIED MATERIALS ON THE DEVELOPMENT OF THE RUMINANT STOMACH

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SUMMARY

Young dairy calves equipped with rumen cannulae were fed on milk and housed on elevated wood platforms. Various materials such as plastic sponges, cellulose sponges, a purified diet, solutions of the sodium salts of acetic, propionic, butyric, and lactic acids were introduced through the cannulae for periods up to 12 wk. The presence of plastic sponges alone did not elicit development of the rumen papillae. The rumens of calves receiving either the purified diet or the fatty acid solutions, with or without sponges, showed marked development.

These data confirm the view that end-products of rumen fermentation rather than the coarse nature of the feed are the stimuli for the development of rumen papillae.

While it has long been recognized that the forestomach of the newborn ruminant is undeveloped, and undergoes a marked change as the animal grows to maturity, relatively little is known of the specific stimuli responsible for this development. In recent reports *(6, 7)*, it was noted that the amount of fat-free dry tissue of the reticulo-rumen and omasum were essentially the same per unit of empty body weight for calves receiving either grain or hay as the only dry feed. The volume displacement of these organs was decidedly larger per unit of empty body weight for the hay-fed calves. The capacity and tissue deposition were much smaller in calves confined on wooden platforms and fed only milk. This lead the authors to postulate that dry feed per se was essential for normal forestomach development, while the bulky nature of hay merely served to stretch these compartments. They further suggested *(3, 4, 6, 7)* that forestomach tissue development, particularly papillary growth, was a result of chemical stimuli, most probably end-products of rumen fermentation, rather than factors of a physical nature.

This latter thesis was also suggested independently by Brownlee *(1)*, who observed marked papillary growth of the rumens of calves bedded on peat moss and receiving high-energy feeds such as concentrates and mangels. He suggested that the amount of the absorbable fraction of the diet was more critical in causing rumen papillae to develop than was the texture of the feed.

The following study was initiated in an attempt to obtain further data as to the factors responsible for stimulating forestomach development.

EXPERIMENTAL PROCEDURES

The experiments were planned on the premise that if chemical entities are responsible for the development of the forestomach, materials of a completely

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soluble nature or of low fiber content should result in the development of rumen papillae and an increase in forestomach tissue. Similarly, if feed texture is of little importance, inert, coarse material would not be effective in promoting the above changes.

Grade male Holstein bull calves were purchased at local auctions as "bob veals". All were fitted with cannula type fistulas (inside diameter $\frac{5}{8}$-in.) at about 2–3 wk. of age, according to the method of Dougherty *(2)*. At about 1 mo. of age, they were placed on the experiment. The calves were confined in tie stalls equipped with elevated wooden floors, to eliminate bedding consumption, and received Holstein milk fed by nipple pail. The amount of milk offered was adjusted periodically, so that all calves made similar gains during the experimental period. Iodized salt was allowed free choice. A mineral supplement and vitamin D were provided, as previously reported *(6)*. Calves were weighed weekly and records of feed consumption were kept.

In planning the treatments, it was felt that if highly soluble materials stimulate forestomach development, their effectiveness would be directly related to the amount offered and the length of time they remained in the rumen. In the following experiments, therefore, some of the test materials were offered several times daily and the amount was increased as rapidly as the calves would adjust to the increases. This fact accounts for the dissimilarity in the treatment pattern for the different calves.

Experiment I. This was an exploratory study to screen the effectiveness of several different materials when introduced through a rumen cannula. The respective treatment materials were as follows:

(1) *Purified diet.* The purified diet was composed of crude casein 25, ground corn starch 50, cerelose 18, and minerals 7. The mineral mixture was a slightly modified synthetic alfalfa ash mixture suggested by Tillman *et al. (5)*. The diet was formulated to have a proximate composition, with the exception of crude fiber and ether extract, similar to the dry calf starter fed in earlier studies *(3, 6)*. The composition was: moisture 14.6%, protein 21.5%, ether extract 0.8%, ash 7.2%, crude fiber 1.2%, and nitrogen-free extract 54.7%.

(2) *Cellulose sponges.* Cellulose sponges having an approximate volume of 186 ml. each were purchased from a local grocery.

(3) *Plastic sponges.* Polyvinyl plastic sponges[2] having an approximate volume of 186 ml. each were administered.

(4) *Fatty acids.* A solution of sodium acetate, sodium propionate, sodium lactate, and sodium butyrate[3] was used.

(5) *Rumen liquor.* Rumen liquor was obtained daily by straining the rumen contents of two fistulated steers through two layers of cheese cloth. The solids

[2] Manufactured in Western Germany and distributed to the chain variety stores by the Ross Distributing Co., New York, N. Y.

[3] Fourteen liters of the stock solution contained: 2,391 g. sodium acetate (Mallinckrodt); 908 g. sodium propionate (Commercial Solvents, Inc.); 875 g. sodium butyrate (Amend Drug & Chemical Co.); 719 g. of a 60% solution of sodium lactate (Merck & Co.); 150 ml. concentrated HCl were added to bring the pH to 6.6. When calculated on the basis of free acids, each liter contained 250 g. in the ratio of 50,20,20,10, respectively.

were returned to the rumen. The steers received 4 lb. alfalfa hay, 14 lb. timothy hay, and 4 lb. of a commercial 14% protein dairy feed daily.

The above treatment materials were administered to the calves through their rumen cannulae as follows: (No. 1)—Six cellulose sponges per week, plus the purified diet. The latter was started at a level of 0.2 lb. per day and increased to a level of 2.4 lb. per day, introduced three times daily. Two hundred milliliters of rumen liquor were given daily. (No. 2)—Six plastic sponges per week, plus the purified diet. The calf was fed increasing amounts of the diet until, just before slaughter, it was receiving 3.3 lb. per day in equal portions three times daily. Two hundred milliliters of rumen liquor were given daily. (No. 3)—Purified diet plus 200 ml. rumen liquor daily. The maximum daily administration of the purified diet was 2.7 lb., administered in three equal portions. (No. 4)—Six plastic sponges the first day of treatment, plus the fatty acid solution. The stock fatty acid solution was diluted with warm tap water such that either one, two, or three liters of liquid was introduced each time and the concentration of acid equivalent never exceeded 50 g. per liter. Supplementation was begun at a level of 10 g. of acids twice daily, and was increased until 120 g. were given three times daily. (No. 5)—Six plastic sponges the first day of treatment, plus rumen liquor. The rumen was filled to capacity twice daily for 8 wk. and three times daily for the last 4 wk. As much as 14 lb. was introduced three times daily during the last week of the experimental period. (No. 6)—Six plastic sponges were introduced weekly.

All calves were slaughtered after 84–91 days of treatment, except No. 1, which was sacrificed after 56 days. The use of some rumen liquor for Calves 1 to 3 was intended to insure the presence of an adequate rumen flora. A few sponges were placed in the rumens of Calves 4 and 5, in an attempt to hold the liquids in the compartment for a longer period of time. Records of treatment materials offered, and body weights, were kept.

Experiment II. Because of pronounced tissue development in the calf receiving fatty acids (No. 4), an additional experiment was initiated. Papillary development of No. 2 was also quite pronounced and many of the sponges found in the rumen had become macerated. The sponges of Experiment II were, therefore, partially ground.

Six Holstein bull calves were used in this experiment and were handled as in Experiment I with regard to purchase, diet, management, and fistulation. They were allotted to three experimental groups, with two calves per group. The treatments were as follows: (1) Fatty acid solution, administered as for Calf No. 4 above; (2) plastic sponges having an approximate volume of 224 ml. each, which had been ground through a ¼-in. hammer mill screen. The number of sponges administered per week were as follows: Weeks 1 and 2, 6; 4–8, 12; 9–12, 24; (3) Treatments 1 and 2 combined. All calves were slaughtered after 85–88 days on treatment, except No. 11, which died of severe gastro-enteritis after 72 days. Pertinent feed, treatment materials, and body weight records were kept as for Experiment I.

During and after slaughter, measurements of volume displacement and tissue fat-free dry matter were made for each calf, as previously described *(6)*. In

addition, the volume of the trimmed, washed, collapsed reticulo-rumen was determined by water displacement. A 4- by 4-in. section of the ventral portion of the dorsal-cranial sac was removed and the mucosal layer containing the papillae separated from the muscle layer. These separate portions were dried at 90° C., allowed to come to equilibrium with the atmosphere for 24–48 hr., and each layer weighed. The resulting per cent mucosa was then calculated. This particular section of the rumen was selected for study, because it was noted earlier *(6)* that it was always the site of greatest papillary growth.

RESULTS AND DISCUSSION

Although the second experiment was conducted as a consequence of data obtained in Experiment I, the results of both studies were in agreement and are discussed together. A summary of the data collected during the treatment period including age, length of treatment period, rate of gain, and intake of milk and treatment materials is shown (Table 1). Results of the slaughter observations are summarized in Table 2.

All calves grew rather well during the experimental period, as evidenced by the average daily gain (Table 1). The attempt to maintain a uniform slaughter weight succeeded fairly well, since the empty body weights of all calves were similar (Table 2). Calf No. 1 was slaughtered after 8 wk. of treatment for preliminary observation and was consequently smaller. The treatments were well-tolerated, although the fatty acids and purified diet tended to be somewhat laxative. Calves apparently utilized the diet, since No. 2 and No. 3 consumed less milk and made somewhat faster gains than did No. 5 and No. 6.

In almost all cases, the plastic sponges from the calves in Experiment I were recovered intact. Calf No. 2 had apparently ruminated some of them, since a few were found macerated in the rumen. The cellulose sponges were partially decomposed and many had disappeared.

In Experiment II, all calves ruminated the ground sponges, usually commencing as soon as they were placed in the rumen. Sponges were found in the feces. At slaughter, the reticulo-rumens were practically free of sponges, except for one calf that had been dosed 48 hr. prior to slaughter.

The various treatments had a markedly different effect on the development of the rumen mucosa. The per cent of the dry weight of the rumen wall composed of mucosa is shown (Table 2). The value was decidedly higher for calves receiving either the purified diet or the salts of the fatty acids, compared with those receiving either plastic sponges alone or rumen liquor. This fact is clearly discernible from the pictures in Figure 1. These photographs are all of the same magnification, and are focused on the area from which the percentage mucosal figures above were derived. The development of papillae was most extensive on the purified diet (No. 3). The fatty acid treatment (No. 7) and the fatty acid plus plastic sponges (No. 11) resulted in definite papillary growth. Neither plastic sponges which remained in the rumen for the entire experiment (No. 6), nor ground plastic sponges which passed fairly quickly through the rumen (No. 9), induced any marked growth. Rumen liquor (No. 5) likewise was ineffective. The percentage mucosa figures were higher for calves receiving the fatty acid salts

TABLE 1

Experimental details of milk-fed dairy calves receiving various purified materials through a rumen cannula

Calf No.	Treatment	Age at slaughter	Length of treatment period	Data collected during treatment period					
				Average daily gain	Total intake of feed and treatment materials				
					Milk	Purified diet	Volume of sponges	Fatty acids[b]	Rumen liquor
		(day)		*(lb.)*			*(liter)*	*(kg.)*	
1	Purified diet plus cellulose sponges	90	56	1.07	487	54	8.9	.....	10.4
2	Purified diet plus plastic sponges	115	86	1.20	602	158	13.4	.....	17.2
3	Purified diet	114	84	1.24	726	122	.....	.....	16.0
7	Salts of fatty acids	123	86	1.07	1014	.....	.....	16.2	.....
8	Salts of fatty acids	117	88	0.91	1042	.....	.....	16.0	.....
6	Whole plastic sponges	119	91	1.03	1042	.....	13.4	.....	.....
9	Ground plastic sponges	123	86	1.23	1346	.....	38.9	.....	.....
10	Ground plastic sponges	121	87	1.08	1172	.....	38.9	.....	.....
4	Fatty acids plus whole plastic sponges	115	87	0.97	847	.....	1.1	15.8	.....
11[a]	Fatty acids plus ground plastic sponges	108	72	1.39	911	.....	32.3	12.8	.....
12	Fatty acids plus ground plastic sponges	123	85	1.20	1082	.....	38.9	16.0	.....
5	Rumen liquor	119	91	1.01	1001	.....	1.1	.....	1,160

[a] Died from gastro-enteritis on the 72nd day of treatment.

[b] Free acid equivalent of the sodium salts actually administered.

TABLE 2

Data obtained following slaughter of milk-fed dairy calves receiving various purified materials through a rumen cannula

Calf No.	Treatment	Empty body weight	Rumen mucosa[b]	Fat-free dry matter[a]			Volume displacement[a]			
				Reticulo-rumen	Omasum	Abomasum	Filled reticulo-rumen	Reticulo-rumen tissue	Omasum	Filled abomasum
		(kg.)	*(%)*	*(g.)*			*(liter)*			
1	Purified diet plus cellulose sponges	71.3	79.9	497	55	48	31.2	3.7	.37	4.6
2	Purified diet plus plastic sponges	89.9	81.2	304	40	64	33.7	3.8	.42	4.2
3	Purified diet	88.9	66.8	341	40	66	41.4	3.7	.51	4.6
7	Salts of fatty acids	91.2	51.1	152	35	79	35.2	1.9	.31	6.8
8	Salts of fatty acids	85.4	53.1	150	73	68	38.7	2.2	.79	7.9
6	Whole plastic sponges	82.2	35.6	196	20	48	30.4	2.2	.15	5.5
9	Ground plastic sponges	99.9	46.5	106	31	68	21.3	1.6	.35	5.9
10	Ground plastic sponges	95.3	34.9	119	29	44	22.2	1.7	.37	3.7
4	Fatty acids plus whole plastic sponges	87.2	65.2	174	40	81	27.3	1.8	.26	3.1
11[c]	Fatty acids plus ground plastic sponges	94.4	63.2	145	44	84	20.2	1.8	.40	.....
12	Fatty acids plus ground plastic sponges	90.3	60.9	163	35	73	35.1	2.1	.59	5.9
5	Rumen liquor	86.7	35.7	136	22	81	27.3	1.6	.18	4.2

[a] Calculated to 100 kg. of empty body weight.
[b] Determined from a 4 sq. in. section removed from the ventralmost portion of the dorsal cranial sac.
[c] Died from gastro-enteritis on the 72nd day of treatment.

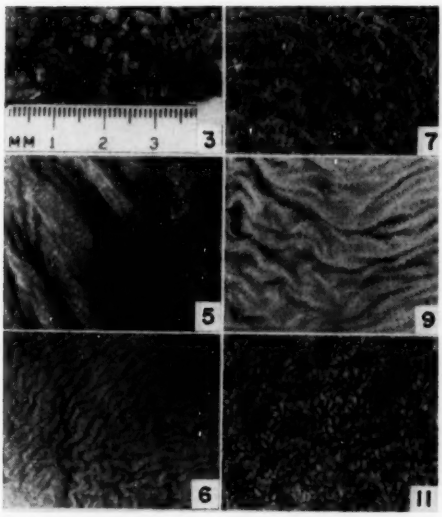

FIG. 1. Photographs of the rumen wall showing the ventralmost portion of the cranial dorsal sac just caudal to the rumino-reticular fold. The treatments of the animals were (3) purified diet, (5) rumen liquor, (6) whole plastic sponges, (7) volatile fatty acids, (9) ground plastic sponges, and (11) volatile fatty acids and ground plastic sponges.

with sponges (Nos. 4, 11, and 12) than for the fatty acids alone (Nos. 7 and 8). Conceivably, the bulky nature of the sponges could have been responsible. In light of the low values for sponges alone (Nos. 6, 9, and 10), a more likely explanation appears to be that the sponges acted as an absorbant, enabling the fatty acid salts to remain in contact with the rumen wall for a longer period of time.

The fat-free dry tissue of the reticulo-rumen when expressed per unit of empty body weight was increased only for the calves receiving the purified diet. These were the only animals that showed appreciable papillary development over the entire rumen surface. The papillae for those calves on the fatty acid treatments were developed in a rather small area in the ventral part of the dorsal cranial sac. The amount of tissue was greatest for the calf on the purified diet plus cellulose sponges, and may be a result of the fermentation of the sponges. The amount of fat-free dry tissue of the omasum and abomasum was not consistently affected by treatment.

The volumes of the filled organs likewise were not materially altered by treatment. The volume of trimmed, washed, collapsed reticulo-rumen tissue was greatest for the calves receiving the purified diet. This result is a reflection of their general increased papillary development.

The volumes of the filled reticulo-rumens are of further interest. In an earlier publication *(6)* it was pointed out that the volumes of the reticulo-rumens of milk-fed calves from birth to 13 wk. of age were about six to eight liters per 100 kg. of empty body weight. [These observations were confirmed by Flatt *(3)*.]

In this same paper *(6)*, it was reported that two milk-fed calves, slaughtered at 16 wk. of age, had reticulo-rumen volumes of 20.7 liters per 100 kg. empty body weight. It was suggested that poor measurement technique could have resulted in the higher value. The reticulo-rumen volumes of the calves in the present study ranged between 20.2 and 41.4 liters per 100 kg. empty body weight. In many of these cases there was little or no papillary development, and the amount of fat-free tissue per unit weight was low. Since all of these calves were over 4 mo. of age, it appears that the capacity of the reticulo-rumen may increase in proportion to body weight to 3-4 mo. of age, but then grow at a more rapid rate thereafter. This observation needs further study.

An explanation for the lack of papillary development in the calf (No. 5) receiving rumen liquor could be that the concentration of stimulating materials was not sufficient to elicit a response. Representative samples of the rumen liquor contained about 2% dry matter. Rumen liquor from animals on a similar diet was assayed for volatile fatty acids and found to contain about 700 mg. per 100 ml. *(3)*. When this value is applied to the amount of rumen liquor administered, the total fatty acids probably did not exceed 8 kg. This value is less than the 16 kg. of the fatty acids given as salts. It is apparent that the failure of Flatt *(3)* to obtain significant papillary development using solutions of fatty acids was due to the low levels employed. He administered a total of 2.5 kg. over a 12-wk. period.

The above data strongly indicate that the most important stimulus for the development of rumen papillae is an active rumen fermentation. The end-products of this fermentation, rather than the coarse nature of the feed, appear to be the specific stimulating entities.

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SHORT-INTERVAL MILKING AS A PHYSIOLOGICAL TECHNIQUE. I. EFFECT OF FREQUENT MILKING WITH THE AID OF OXYTOCIN ON MILK AND MILK FAT PRODUCTION [1]

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SUMMARY

Thirteen Holstein cows at various stages of lactation and levels of production were milked at 2-hr. intervals with the aid of oxytocin. It was shown that the average daily milk production was increased and that the fat per cent in milk was reduced for the high producers (average daily production: 60.6 lb. milk) during the 2-hr. milking. Following return to twice-a-day milking, the milk production returned to normal, but there was an over-compensatory increase in fat per cent. The moderate producers among the cows (average daily production 39–43 lb. milk) did not show any significant changes in milk production, fat production, or fat per cent. It is concluded that the rate of milk secretion is not altered significantly for moderate producers on changing from twice-daily milking to 2-hr. milking for short total periods. The response of the high producers is explained on the basis of the effects of intramammary pressure on fat uptake by the mammary gland and on the rate of milk secretion.

The study of the physiological kinetics of milk secretion with the aid of radioactive tracers makes it necessary to milk cows at short intervals. Such milking can be done routinely only with the aid of oxytocin injections to obtain ejection of milk. Since the postulation of the neurohumoral hypothesis of milk ejection *(5)*, oxytocin has been widely used to obtain experimental ejection of milk. However, no attempt has been made to evaluate short-interval milking as an experimental approach to problems in milk secretion.

The present series of papers is the result of a study made to develop and apply short-interval milking as a physiological technique. In the course of this work, data were collected on the effect of repeated milking with the aid of oxytocin on milk and fat production. In view of the existing conflict in the literature, it seems advisable to publish these data.

Shaw *(10)* milked cows at 2-hr. intervals for periods up to 40 hr. and found that milk yields and fat yields were relatively uniform and that, while milk yields were not affected by oxytocin injections, fat yields and fat per cent in milk were lowered significantly. Smith *(13)* confirmed the uniformity of milk yields and fat yields and showed that milking at 2-hr. intervals, with the aid of oxytocin, reduces greatly the variability normally found in these. Smith, however, reported that both milk yields and fat yields were slightly increased during 2-hr. milking, although the fat per cent in milk was lowered. He also recorded an increase in

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fat per cent in milk after the experimental period of 2-hr. milking. During this period, milk and milk fat production were slightly lowered. Donker *et al. (2)* have shown more recently that when cows are milked at hourly intervals there is an initial depression in fat secretion, followed by a marked increase.

EXPERIMENTAL PROCEDURE

The data reported in this paper are from two experiments using Holstein cows. Nine cows were selected for the first experiment from the Beltsville Experiment Station herd to represent a wide range in production (30–70 lb. milk daily) and stages of lactation (1–7 mo. in milk). The routine feeding and management regime of these cows was not changed materially when they were placed on experiment, except for a decrease in the hay intake, to maintain a greater uniformity and adequacy of the energy intake. All feed was weighed during a ten-day preliminary period and during the remainder of the experiment to maintain the cows on a constant feeding regime throughout the study. This was established at Morrison's *(9)* recommended total digestible nutrient intake in three-day tests prior to the preliminary period. U. S. No. 2 leafy alfalfa hay was fed at a level of 1.5 lb. per 100 lb. of body weight per day and a 17% protein concentrate mixture provided the remainder of the desired intake of total digestible nutrients (TDN). The average for daily milk and fat production for the last three days of the preliminary period constitutes the production data presented for the preliminary period, or Period I.

During the experimental period (or Period II), the cows were milked by hand at 2-hr. intervals for 18 consecutive milkings. The intervals between milkings were never less than 115 min. and never more than 130 min. Immediately before each milking the cows received an injection of 10 I.U. of oxytocin.[5] The first injection was made at 6 A.M. one morning, 2 hr. after the last normal herd milking. The last injection was made at 4 P.M. the next day, 34 hr. later. The milk samples were weighed and analyzed for fat individually. The data presented for Period II are taken from the last 12 milkings for this period, the first six being discarded as representing a transition.

Following this experimental period, the cows were returned to normal twice-a-day milking under herd conditions. Records of milk weights and fat tests were kept on individual milkings for the next six days. The day after the experimental period was regarded as a period of adjustment and the data presented for the postexperimental period, or Period III, are the average for the next five days.

To complement the data obtained from the first experiment, a second experiment was conducted later (December), using four cows that were moderate producers (34–49 lb. milk per day) in relatively early lactation (2–5 mo. in milk). The preliminary adjustment period was increased to 15 days. The milk production data for five days prior to the experimental period were used as data for the preliminary period, or Period I. The experimental procedure was otherwise the same as in the first experiment.

[5] Squibb, obstetrical pituitrin.

The milk yields were corrected to 4% fat-corrected milk (4% FCM) according to Gaines *(6)*. Analyses of variance were performed according to accepted methods *(14)* and the new multiple range test of Duncan *(3)* was chosen, to arrive at the significance of the difference between the means.

RESULTS

The data for the average milk yields, fat yields, 4% corrected milk, and the weighted average fat per cent in milk for all of the cows in the two experiments during the three periods of study are found in Table 1. Striking differences could be noticed in the response of five cows in the first experiment that could be classified as high-producing cows compared to the remaining four which were lower producers. Therefore, the data for the two groups of cows in the first experiment are reported separately. The analyses of variance (Table 2) are also reported separately, for these two groups as well as for the two experiments. All statements on the significance of the differences between means are based on the application of Duncan's test *(3)*.

Milk production. All cows increased somewhat in milk production when milked at 2-hr. intervals (Period II). The milk production was also slightly higher in the postinjection period (Period III) for nine of the 13 cows. However, the most marked response was to be found in the five high producers in early lactation which formed the first group of the first experiment. This is reflected in the F values in Table 2. It was shown by Duncan's test that the differences in milk production between Periods II and I and between Periods II and III were significant at a protection level of $P = 0.01$ for this group of five cows. The differences in milk production between periods for the second group of cows in the first experiment, as well as for the cows in the second experiment, were not significant.

Fat production. Fat production remained relatively uniform during Period II as compared with Period I. In some of the cows, this was because an increase in milk production was balanced by a decrease in fat per cent. In others, this was because there was no significant change in either. During Period III, the mean fat production was slightly increased in all groups. However, none of the changes observed in fat production was significant at the $P = 0.05$ level.

Fat per cent in milk. Fat per cent, on the whole, was lower in Period II than in Period I. This, again, was most noticeable in the first group of the five high-producing cows in the first experiment. Following this, in Period III, the fat per cent was increased even beyond the level in Period I.

The depression in fat per cent during Period II was significant at the protection level of $P = 0.05$ for the first five cows of the first experiment. The increase in fat per cent from Period II to Period III was significant at the $P = 0.01$ level. The difference in fat per cent between Periods III and I was not significant. For the other four cows of this experiment, as well as for cows in the second experiment, none of the changes observed in fat per cent was significant at the $P = 0.05$ level.

fat per cent in milk after the experimental period of 2-hr. milking. During this period, milk and milk fat production were slightly lowered. Donker *et al. (2)* have shown more recently that when cows are milked at hourly intervals there is an initial depression in fat secretion, followed by a marked increase.

EXPERIMENTAL PROCEDURE

The data reported in this paper are from two experiments using Holstein cows. Nine cows were selected for the first experiment from the Beltsville Experiment Station herd to represent a wide range in production (30–70 lb. milk daily) and stages of lactation (1–7 mo. in milk). The routine feeding and management regime of these cows was not changed materially when they were placed on experiment, except for a decrease in the hay intake, to maintain a greater uniformity and adequacy of the energy intake. All feed was weighed during a ten-day preliminary period and during the remainder of the experiment to maintain the cows on a constant feeding regime throughout the study. This was established at Morrison's *(9)* recommended total digestible nutrient intake in three-day tests prior to the preliminary period. U. S. No. 2 leafy alfalfa hay was fed at a level of 1.5 lb. per 100 lb. of body weight per day and a 17% protein concentrate mixture provided the remainder of the desired intake of total digestible nutrients (TDN). The average for daily milk and fat production for the last three days of the preliminary period constitutes the production data presented for the preliminary period, or Period I.

During the experimental period (or Period II), the cows were milked by hand at 2-hr. intervals for 18 consecutive milkings. The intervals between milkings were never less than 115 min. and never more than 130 min. Immediately before each milking the cows received an injection of 10 I.U. of oxytocin.[5] The first injection was made at 6 A.M. one morning, 2 hr. after the last normal herd milking. The last injection was made at 4 P.M. the next day, 34 hr. later. The milk samples were weighed and analyzed for fat individually. The data presented for Period II are taken from the last 12 milkings for this period, the first six being discarded as representing a transition.

Following this experimental period, the cows were returned to normal twice-a-day milking under herd conditions. Records of milk weights and fat tests were kept on individual milkings for the next six days. The day after the experimental period was regarded as a period of adjustment and the data presented for the postexperimental period, or Period III, are the average for the next five days.

To complement the data obtained from the first experiment, a second experiment was conducted later (December), using four cows that were moderate producers (34–49 lb. milk per day) in relatively early lactation (2–5 mo. in milk). The preliminary adjustment period was increased to 15 days. The milk production data for five days prior to the experimental period were used as data for the preliminary period, or Period I. The experimental procedure was otherwise the same as in the first experiment.

[5] Squibb, obstetrical pituitrin.

The milk yields were corrected to 4% fat-corrected milk (4% FCM) according to Gaines *(6)*. Analyses of variance were performed according to accepted methods *(14)* and the new multiple range test of Duncan *(3)* was chosen, to arrive at the significance of the difference between the means.

RESULTS

The data for the average milk yields, fat yields, 4% corrected milk, and the weighted average fat per cent in milk for all of the cows in the two experiments during the three periods of study are found in Table 1. Striking differences could be noticed in the response of five cows in the first experiment that could be classified as high-producing cows compared to the remaining four which were lower producers. Therefore, the data for the two groups of cows in the first experiment are reported separately. The analyses of variance (Table 2) are also reported separately, for these two groups as well as for the two experiments. All statements on the significance of the differences between means are based on the application of Duncan's test *(3)*.

Milk production. All cows increased somewhat in milk production when milked at 2-hr. intervals (Period II). The milk production was also slightly higher in the postinjection period (Period III) for nine of the 13 cows. However, the most marked response was to be found in the five high producers in early lactation which formed the first group of the first experiment. This is reflected in the F values in Table 2. It was shown by Duncan's test that the differences in milk production between Periods II and I and between Periods II and III were significant at a protection level of $P = 0.01$ for this group of five cows. The differences in milk production between periods for the second group of cows in the first experiment, as well as for the cows in the second experiment, were not significant.

Fat production. Fat production remained relatively uniform during Period II as compared with Period I. In some of the cows, this was because an increase in milk production was balanced by a decrease in fat per cent. In others, this was because there was no significant change in either. During Period III, the mean fat production was slightly increased in all groups. However, none of the changes observed in fat production was significant at the $P = 0.05$ level.

Fat per cent in milk. Fat per cent, on the whole, was lower in Period II than in Period I. This, again, was most noticeable in the first group of the five high-producing cows in the first experiment. Following this, in Period III, the fat per cent was increased even beyond the level in Period I.

The depression in fat per cent during Period II was significant at the protection level of $P = 0.05$ for the first five cows of the first experiment. The increase in fat per cent from Period II to Period III was significant at the $P = 0.01$ level. The difference in fat per cent between Periods III and I was not significant. For the other four cows of this experiment, as well as for cows in the second experiment, none of the changes observed in fat per cent was significant at the $P = 0.05$ level.

TABLE 1

The milk and fat production for the 13 cows used in the study[a]

Cow No.	Months in milk	Milk						Fat			4% FCM		
		I	II	III	I	II	III	I	II	III	I	II	III
		(lb.)			(% fat)			(lb.)					
					Cows in first experiment—first group								
2039	1	75.3	83.3	79.7	4.21	3.52	4.79	3.17	2.92	3.82	77.7	77.1	89.2
2076	2	58.7	63.8	58.5	4.33	3.46	4.30	2.54	2.21	2.52	61.6	58.7	61.2
1672	3	57.9	65.6	58.5	3.70	3.16	4.20	2.14	2.07	2.46	55.3	57.3	60.3
2092	3	57.7	63.3	58.7	3.45	3.29	4.19	1.99	2.08	2.46	52.9	56.5	60.4
1688	3	53.2	64.3	54.9	3.83	3.17	3.56	2.04	2.04	1.98	51.9	56.3	51.7
Mean:		60.6	68.1	62.1	3.93	3.32	4.25	2.38	2.26	2.64	59.9	61.2	64.6
					Cows in first experiment—second group								
2079	6	48.8	51.0	51.3	3.44	3.16	4.21	1.68	1.61	2.16	44.7	44.6	52.9
2459	5	37.7	40.0	37.1	3.69	4.23	4.56	1.39	1.69	1.69	35.9	41.4	40.2
2407	6	37.7	38.5	36.9	4.72	4.16	5.17	1.78	1.60	1.91	41.8	39.4	43.4
1655	7	35.2	37.2	38.7	4.06	3.84	3.56	1.43	1.43	1.38	35.5	36.3	36.2
Mean:		39.9	41.7	41.0	3.94	3.79	4.34	1.57	1.58	1.78	39.4	40.4	43.2
					Cows in second experiment								
2056	4	49.3	53.1	50.2	3.61	3.54	3.95	1.78	1.90	1.98	46.4	49.7	49.8
2463	2	48.9	50.7	48.3	3.79	3.79	4.25	1.85	1.93	2.03	47.3	49.2	49.8
2423	3	38.0	38.7	39.3	4.07	3.94	3.75	1.55	1.84	1.47	38.5	38.6	37.8
2095	5	34.6	37.2	36.6	3.16	3.03	3.47	1.09	1.13	1.27	30.2	31.9	33.7
Mean:		42.7	44.9	43.6	3.68	3.63	3.88	1.57	1.63	1.69	40.6	42.4	42.8

[a]Period I: Preliminary period of normal herd milking (twice-a-day); Period II: The injection period of milking with the aid of oxytocin at 2-hr. intervals; Period III: The postinjection period of twice-a-day herd milking.

TABLE 2

Mean squares (M.S.) and variance ratios (F) of the data on two experiments on short-interval milking (from Table 1)

Source of variance	Degrees of freedom	Milk production		Fat per cent		Fat production		4% FCM	
		M.S.	F	M.S.	F	M.S.	F	M.S.	F
Cows in first experiment—first group									
Between cows	4	240.95	121.0[a]	0.2306	3.83	0.7783	19.26[a]	374.9	31.9[a]
Between periods	2	78.75	33.7[a]	1.018	16.9[a]	0.1950	4.83[b]	29.18	2.49
Error	8	2.34		0.0602		0.0404		11.74	
Cows in first experiment—second group									
Between cows	3	121.73	79.6[a]	0.6645	3.95	0.1002	3.48	70.28	10.37[a]
Between periods	2	3.36	2.19	0.3022	1.79	0.0583	2.02	15.09	2.23
Error	6	1.53		0.1684		0.0288		6.78	
Cows in second experiment									
Between cows	3	165.3	179.1[a]	0.338	8.99[a]	0.390	79.88[a]	204.98	181.2[a]
Between periods	2	5.01	5.4[b]	0.0828	2.2	0.0145	2.97	5.32	4.71
Error	6	0.923		0.0376		0.00488		1.13	

[a] Significant at the 1% level.
[b] Significant at the 5% level.

Four per cent FCM. The mean production of 4% FCM was slightly increased during Period II and increased further in Period III. While this was true for all groups of cows, none of the changes observed was significant at the $P = 0.05$ level.

No correlation could be established between the changes in milk production (magnitude or percentagewise) and the corresponding changes in fat per cent.

DISCUSSION

The 13 cows in the two experiments can be grouped conveniently into three categories. Five high-producing cows in early lactation and four medium-producing cows in later lactation comprised the first experiment. Four medium producers in early lactation were used in the second experiment. The foregoing results may be summarized for discussion as follows: The cows in the first category (high producers in early lactation) showed a marked increase in milk production during short-interval milking (Period II), accompanied by a depression in the fat per cent. On returning the cows to twice-a-day herd milking (Period III), the milk production and fat per cent returned to the original levels. In the case of the fat per cent, a certain measure of over-compensation could be discerned. The cows in the other categories, while they tended to reflect these trends somewhat, did not show any statistically significant changes. Duncan's multiple range test, which tends to give a very conservative estimate of the significance of the differences between means, was used to estimate the significance of the differences between the means for various periods for the cows in the first category. The differences between the means of various periods for the other cows were shown not to be significant, even by the variance ratio (F test), which may be regarded as one of more liberal estimates of such significance. Hence, the conclusions stated above have adequate statistical basis.

One may rule out seasonal factors or variations in energy intake as being causes for this difference. The cows were stall-fed 100% of Morrison's recommended standard during the preliminary and experimental periods. The two categories of cows in the first experiment received identical preliminary treatment. The stage of lactation, too, appears unlikely to be a factor, since cows in early lactation in the second experiment did not show the increase in milk production or decrease in fat test shown by the high producers in Group 1.

It should be mentioned that the production of 4% fat-corrected milk was not altered for any of the three categories of cows, during or after the short-interval milking, indicating no particular stimulus to the gland. The two factors of the anterior pituitary, namely ACTH and STH, that do affect milk and fat secretion rapidly and markedly do not affect the same type of changes found in the present data (for a review, see Reference *11*). It is not necessary at the present state of our knowledge to postulate any new humoral factor, especially since it is possible to explain the findings with some accepted concepts as to the course of milk secretion.

The concept that the rate of milk secretion depends on intramammary pressure and, hence, to a certain extent on the milking interval, is not new. The

advantage of reduced intramammary pressures would be proportionately greater for high producers. Hence, by far the greatest reason for the difference in response shown by the five high producers would appear to be the level of production. It should be possible to confirm this by using high producers in late lactation and it is to be hoped that the present data will spur others to test the validity of this conclusion. It must be realized that tacit assumptions on the capacity of the alveolar lumina are involved in both of the above conclusions. Similar assumptions are involved in the statement by Edwards *(4)*, that the effect of pressure on secretory activity is probably a function of the gland capacity and stage of lactation rather than function of time. The importance of further verification of these assumptions by the present experimental approach, as well as by other independent means, can not be overemphasized. A detailed evaluation of our present ideas as to the course of lactation should logically follow a discussion of the variations in milk yields and fat yields during 2-hr. milking and, hence, will be made in the succeeding paper of this series.

Shaw and Petersen *(12)* have shown that the distention of the alveoli is necessary for the normal passage of fat into the gland, and that the fat uptake by the gland was inhibited by removal of milk from the gland. This, indeed, was the rationale of Shaw's original work *(10)* on 2-hr. milking. The depression in fat production found by him, and the increase in milk production only, as contrasted with no increase in fat production found by Smith *(13)*, and the similar observation in this paper for one group of cows, would substantiate this theory. Other recent work *(7, 8)* is also confirmatory in effect. If we accept this line of argument, then the maximum depression in fat per cent in milk would be seen in those cows that are high producers and, therefore, tend to have higher sustained intramammary pressure normally.

In essence, the present work is not in substantial disagreement with the work either of Shaw *(10)* or of Smith *(13)*. Indeed, by identifying at least one of the factors that affect the response, namely, the level of production, this paper explains somewhat the discrepancies in the findings of the two workers. Some of the differences in conclusions between the present work and the earlier work might be because the earlier workers neglected to put their animals on controlled feed intake. A second reason might be the more rigid exclusion of transitional milkings in the present paper. The greatest reason, however, may lie in the statistical approach. Shaw's analyses of his data were limited in scope. The rank-order method used by Smith, while it establishes trends, gives an exaggerated estimate of the significance of the trends. Smith's statement on the inapplicability of the conventional statistical methods to problems of the present type is well-founded. But until adequate statistical approaches are designed, it is perhaps safer to err, if at all, on the conservative side.

By far the most significant finding is that, for at least up to 36 hr., cows in moderate production did not exhibit marked changes in milk and fat production during short-interval milking. This would form the basis of the experimental technique for short-interval milking presented in subsequent papers. It also shows that the rate of milk secretion during 2-hr. milking is not significantly

greater for most cows than during twice-daily milking. The conclusion reached by some workers, that such increases are noticeable in cows in moderate production *(1)*, will be discussed in the succeeding paper.

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VARIATION IN THE THYROXINE-SECRETION RATE OF CATTLE[1, 2]

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SUMMARY

A total of 104 individual estimations of thyroxine secretion rates in 29 animals was made in dairy cattle during various months of the year, along with the determinations of the rate constants for hormone release normally and when the recycling of iodine was blocked with thiouracil. The relationship of thyroxine-secretion rates with hormonal release rates was rather poor. This is believed to be due to the variation in the size of the thyroid gland with identical or different release rates. None of the indices of thyroid activity satisfactorily measured the actual amount of thyroxine secretion. The average thyroxine-secretion rate for the cows was 0.56 mg. (range 0.2–1.0), L-thyroxine/100 lb. body weight in winter, and 0.6 mg. (range 0.2–1.0) L-thyroxine/100 lb. body weight for calves during the same period. Thyroxine-secretion rates were reduced threefold in the summer, but the extent of depression varied with the individual. There was an average threefold variation in thyroxine-secretion rate between individuals during the same season. The rate of change in thyroxine secretion was not in direct proportion to the rate of seasonal change under decreasing ambient temperature, and it is thought that an increasingly warm environment may be a greater stimulus to a reduction in thyrotropic hormone secretion than a gradually colder environment for increased thyrotropic activity.

Pipes *et al. (8)* described a technique for determination of the thyroxine-secretion rate in individual dairy animals. The thyroxine-secretion rates have now been determined in a group of heifers, mature cows, and two young bulls during various seasons of the year. In many of these animals, in addition, the normal rate of release of thyroidal-I^{131} and the release rate when a goitrogen was administered also were determined. Since the thyroxine-secretion rate determination requires simultaneous administration of thiouracil and thyroxine for about 2 wk. or longer, it was thought possible that one or the other of these indices of thyroid gland activity might be closely related to the observed rate of thyroxine secretion and, thus, might serve as a substitute for the more laborious thyroxine estimation. As will be shown, the normal release rates of thyroidal-I^{131}, or the release rate when recycling was prevented by goitrogen, were only slightly related to thyroxine-secretion rate. It is believed that only the thyroxine-secretion rate adequately measures thyroid gland function.

The cattle studied were subjected to diurnal and seasonal ambient temperatures prevailing in Missouri. It is recognized that environmental temperature influences thyroxine-secretion rate, but data on cattle using the present method are very limited.

The object of the present paper is to compare thyroxine-secretion rates of cattle estimated at various times of the year with other indices of thyroid gland function, to show the influence of seasonal variation in ambient temperature

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upon thyroxine-secretion rate and, finally, to show the variation in individual thyroxine-secretion rates of cattle during winter and summer periods.

Experimental. The cattle included Jersey, Guernsey, and Holstein heifers and mature cows and bull calves. They were kept in a dry lot except when milked. Hay and a concentrate mixture were fed throughout the year to avoid changing pasture conditions. A block of iodized salt was always available. Between 100–300 μc. of iodine^{131} was injected intravenously into each animal according to body weight. After maximal thyroidal-I^{131} was attained (second or third day), daily measurements of thyroidal-I^{131} were made for a period to obtain the normal rate of release of thyroid hormone (k'_4); then, a goitrogen, thiouracil, was fed by capsule at a rate of 1 g/100 lb. body weight for a period, to obtain the release rate when the recycling of I^{131} was prevented (k''_4). Finally, the thyroxine-secretion rate was determined by the daily injection of increasing amounts of purified L-thyroxine with concurrent administration of thiouracil, the amount being increased every third day until the release of thyroidal-I^{131} was prevented by inhibiting the discharge of the thyrotropic hormone.

RESULTS

Effect of temperature variations on hormone release rates. The normal release rate of thyroid hormone when related to season (average temperature) during the year follows a straight line (Figure 1). This was rather unexpected, since thyroxine-secretion rate showed an inverse relation with season. There were wide variations in the rate constants which apparently were unrelated to temperatures. When the uptake of I^{131} was blocked with thiouracil, the apparent rate of discharge of thyroidal-I^{131} was increased, and the inverse rela-

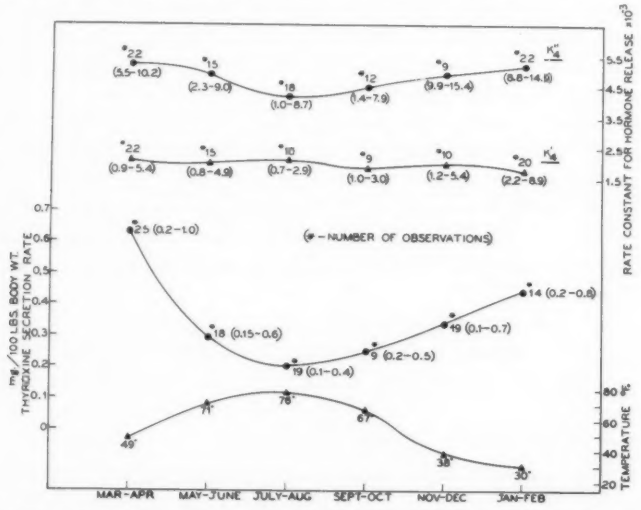

FIG. 1. The seasonal trend in thyroxine-secretion rate is shown in relation to average temperature changes at the Missouri Agricultural Experiment Station.

tionship with season also increased. During the period of observation, the animals were subjected to average temperature variations by as much as 30° F. in January to 78° F. in July.

Thyroxine-secretion rates versus hormone release rates. In an attempt to clarify the relationship between thyroxine-secretion rate and thyroid hormone release rate, it was observed that variations in thyroxine-secretion rate were not accompanied by proportionate variations in the rates of hormone release with or without a goitrogen (Table 1). A gross relationship between the two is apparent, however, if the ranges of comparisons are wide. Small differences in release rates of the hormone are not accompanied by appreciable differences in thyroxine-secretion rates (Table 2). The lack of relationship is believed to be due to differences in thyroid gland size of individual animals. Thus, two thyroid glands might release thyroidal-I^{131} at the same rate, but if one were twice as large as the other it might secrete twice as much thyroxine per day. It is possible that an animal having a small thyroid gland might have a higher release rate, in order to attempt to compensate for the small thyroid gland, but yet might secrete little thyroxine per day.

While diversity in the size of the thyroid glands can be offered as an explanation for the lack of relationship between thyroxine-secretion rate and the hormone release rate between animals, complicated problems are encountered in some animals when the parameters of thyroid activity are compared be-

TABLE 1

Comparison between thyroxine-secretion rates and release rate constants

Thyroxine-secretion rate *(mg/100 lb. body weight)*	No. of observations	Normal rate of release of thyroidal-I^{131} k′4	No. of observations	Release of thyroidal-I^{131} when goitrogen is administered k″4
0.1–0.2	37	0.0023 ± 0.0003	36	0.0050 ± 0.0005
0.2–0.4	9	0.0015 ± 0.0005	3	0.0036 ± 0.0005
0.4–0.6	18	0.0021 ± 0.0003	10	0.0050 ± 0.0006
0.6–0.8	5	0.0040 ± 0.0009	2	0.0070 ± 0.0007
0.8–1.0	1	0.0059	1	0.0073

TABLE 2

Thyroid hormone release rate compared with the thyroxine secretion

Normal rate of release of thyroidal-I^{131} k′4	No. of observations	Thyroxine-secretion rate *(mg/100 lb. body weight)*	Release of thyroidal-I^{131} when goitrogen is administered k″4	No. of observations	Thyroxine-secretion rate *(mg/100 lb. body weight)*
0.0002–0.0010	17	0.37	0.0002–0.0010	2	0.15
0.0011–0.0020	18	0.33	0.0011–0.0020	4	0.15
0.0021–0.0040	25	0.51	0.0021–0.0040	11	0.39
0.0041–0.0060	8	0.80	0.0041–0.0060	17	0.37
0.0061–0.0080	2	0.20	0.0061–0.0080	12	0.63
0.0081–0.0160			0.0081–0.0160	5	0.70

tween summer and winter in the same animal. Apparently, in some individual animals, the rate at which the hormone is released from the thyroid gland does not change with seasons and, furthermore, a slight increase in rate is noted in some individuals during summer (Table 3), but the thyroxine-secretion rate is lower in summer than in winter. Such a situation can be explained if it is assumed that a part of the thyroid gland becomes inactive in summer while the remainder of the gland tries to compensate by an accelerated rate of release of the hormone; but, the absolute amount of hormone secreted is less owing to the functioning of a limited number of cells, which is also reflected in the relatively low percentage of uptake of I^{131} in such animals. Kurland *et al. (4)* have described cases in humans where a small portion of the thyroid gland

TABLE 3

Parameters of thyroid function in individual animals during summer and winter seasons

	Summer				Winter			
Animal No.	I^{131} uptake %	Normal rate of release of thyroidal-I^{131} k'_4	Release of thyroidal-I^{131} when goitrogen is administered k''_4	Thyroxine-secretion rate *(mg/100 lb. body weight)*	I^{131} uptake %	Normal rate of release of thyroidal-I^{131} k'_4	Release of thyroidal-I^{131} when goitrogen is administered k''_4	Thyroxine-secretion rate *(mg/100 lb. body weight)*
24	40.1	0.0100	0.0100	0.28	45.0	0.0092	0.0065	0.6
3625	36.3	0.0016	0.0057	0.10	59.8	0.0019	0.0053	0.4
3626	49.0	0.0020	0.0045	0.20	52.0	0.0019	0.0051	0.6
42	42.2	0.0022	0.0040	0.18	51.1	0.0024	0.0036	0.5
76	35.1	0.0025	0.0045	0.17	40.2	0.0022	0.0047	0.6
Henry 2	50.1	0.0008	0.0042	0.17	40.1	0.0018	0.0047	0.6

works at an increased rate with the effective maintenance of a normal level of serum hormone. It is also possible that there might be significant variations in the activity of follicles dependent upon variation in vascular supply during summer. In rats, the follicles at the periphery of the gland are reported to be less active than central follicles. It is also possible, although unlikely, that some nonthyroidal-I^{131} might diffuse from the thyroid into the blood stream in summer, to enhance the apparent release rates of the hormone. From the above discussion, it is clear that small differences in thyroidal-I^{131} release rates do not measure thyroid function with any degree of accuracy.

This and previous studies are believed to indicate that the following indices of thyroid gland function, namely, I^{131} uptake, normal release rates, or release rates where iodine recycling has been prevented, are unsatisfactory measures of actual thyroxine-secretion rate *(7, 9)*.

Effect of season on thyroxine-secretion rate. A total of 104 individual determinations of thyroxine-secretion rates were made in cows and calves during various months of the year. From a study of the trend in values obtained during various months of the year (Figure 1), the thyroxine-secretion rates determined during the months from December to April were considered as representing winter and those determined from May to September, as summer.

During each season, from two to four determinations of thyroxine-secretion rates were made in each individual animal and the average secretion rate for the winter and summer reveals a threefold seasonal variation in individuals (Table 4). In summer, the thyroxine-secretion rate is depressed in all animals. While the extent of depression is variable, an average of about 30% of winter values is noted in summer.

When thyroxine-secretion rates are plotted against temperature and season, a seasonal change is readily apparent (Figure 1). A similar seasonal variation in the per cent of fat in cow's milk has been noted by Ragsdale and Turner *(10)*. A close scrutiny of the seasonal variation reveals that the rate at which the thyroxine secretion decreased for an increase of 30° temperature (48–78° F.)

TABLE 4

Individual thyroxine-secretion rates of dairy cattle

Breed	Cow-No.	Age	Thyroxine-secretion rate (*mg/100 lb. body weight*)			
			Winter[a]	Range	Summer[b]	Range
		(yr.)	Cows			
Jersey	160	7	0.7	0.60–0.80	0.1	0.10–0.10
	158	4	0.7	0.60–0.80	0.2	0.25–0.20
	159	4	0.7	0.60–0.80	0.2	0.15–0.25
	310	6	0.4	0.25–0.60	0.1	0.10–0.15
	580	6	0.6	0.60–0.70	0.1	0.15–0.05
	585	5½	0.4	0.40–0.25		
	544	7	0.7	0.65–1.00	0.4	0.45–0.35
	601	4	0.3	0.20–0.45	0.15	0.10–0.20
	594	4½	0.5	0.25–0.65	0.15	0.10–0.20
	583	6	0.5	0.35–0.70	0.4	0.35–0.50
	J 1	5	0.6	0.65–0.60		
	82	4	0.6	0.60–0.70		
	348	4	0.3	0.30–0.30		
		Av.	0.56		0.2	
Guernsey	3596	4	0.6	0.60–0.80	0.2	0.25–0.15
	3625	4	0.4	0.20–0.60	0.1	0.10–0.15
	3626	4	0.2	0.20–0.30	0.1	0.20–0.05
		Av.	0.4		0.13	
Holstein	402	7	0.6	0.60–0.60	0.1	0.10–0.15
			Heifers			
			Winter[b]	Range	Summer[b]	Range
Jersey	G41	2½	0.6	0.50–0.60	0.17	0.15–0.20
	G42	2½	0.5	0.40–0.70	0.18	0.10–0.25
	24	2½	0.6	0.60–0.70	0.28	0.20–0.40
	76	2½	0.6	0.60–0.70	0.17	0.15–0.20
	89	1½	0.7	0.65–0.90	0.15	0.15–0.15
		Av.	0.6		0.19	
Guernsey	38	3			0.15	0.10–0.20
	41	3			0.15	0.15–0.10
		Av.			0.15	
Holstein × Jersey heifer		1½	0.4	0.30–0.45		
Jersey bull		3	0.7	0.50–0.85	0.18	0.15–0.20
Holstein × Jersey bull		10 mo.	0.4	0.30–0.55	0.17	0.10–0.25

[a] 3–4 estimations.
[b] 2–3 estimations.

is faster than the rate at which thyroxine secretion increases over the same range of decrease in temperature (78–48° F.). Over the range of temperatures specified, the drop in thyroxine-secretion rate was from 0.63 to 0.2 mg/100 lb. body weight (difference, 0.43 mg.); whereas, the increase was from 0.2 to 0.3 mg/100 lb. body weight (difference, 0.1 mg.). In general, changes in approaching summer (descending segment in the curve, Figure 1) were more rapid than in approaching winter (ascending segment in Figure 1), although the temperature difference was greater in the latter case. Consequently, a time lag was apparent in the elevation of thyroxine-secretion rates from summer to winter.

Individual variation in thyroxine-secretion rate. The average thyroxine-secretion rate of a total of 26 individual animals of different breeds, ages, and sexes has now been determined during the months of December to April (winter). Individual estimations range from 0.2 mg/100 lb. body weight per day to 1.0 mg., while the range in average thyroxine secretion was 0.2 mg. to 0.7 mg/100 lb. body weight per day. It is expected that as thyroxine-secretion rates of more productive cattle are determined, the upper range may be further extended.

Discussion. Although the literature contains a number of reports on the effect of season on thyroid activity in other species, the information on cattle is very limited and somewhat contradictory. Thus, Lodge *et al. (6)* reported lower thyroxine-secretion rates in heifers in summer, but the same group of workers *(5)* in another trial failed to confirm the previous observation. It was suggested that the animals were not exposed to extreme temperature variations, since the animals were confined to the barn. A cool summer season might be a factor, also. Blincoe and Brody *(2)*, using the uptake of iodine as the index of thyroid activity, demonstrated a positive correlation with temperature changes in the psychroenergetic laboratory. Swanson et *al. (12)*, using the same index of thyroid activity, observed little seasonal variation. They suggested that the capacity of the thyroid to incorporate I^{131} was not a sensitive indicator of iodine metabolism or of thyroid activity. Again, Werner *et al. (13)* were not able to demonstrate any seasonal differences in iodine uptake in humans. Although the values for uptake have not been presented in our study, the range was 15–65% of the administered dose of I^{131}. That the uptake of iodine is a poor criterion of thyroid function has been shown by several workers *(7, 12)*. The uptake of iodine is easily altered by circumstances that are within the normal range of thyroid physiology, and interpretation of high or low uptake is always complicated by the effect of exogenous iodine from unidentified sources. Also, changes in renal function and excretion of iodine in milk will certainly influence the iodide pool in the body, to affect the uptake value. The release of I^{131} from the gland is claimed to be a better index of thyroid activity, since it is directly related to hormone secretion and is not affected to any great extent by the factors of renal loss of I^{131} *(11)*, changes in iodide space *(1)*, or blood iodine content *(3)*. Nevertheless, our results fail to show a significant relationship between these release rate constants, season, or thyroxine-secretion rate

(Figure 1, Tables 1 and 2). It is believed that the determination of thyroid activity by substitution therapy with exogenous thyroxine is the best index for quantitative comparisons.

The fact that the rate of change in thyroxine secretion is not in direct proportion to the rate of seasonal change under decreasing ambient temperature suggests that an increasingly warm environment is a greater stimulus to a reduction in thyrotropic hormone secretion than is a gradually colder environment for increased thyrotropic activity.

It is also interesting to speculate on the significance of the variation in the depression of thyroxine-secretion rate between individual cows during summer months. It will be observed that in Cows 583 and 544 (Table 4), the lowering of thyroxine-secretion rate during the hot months is relatively much less than in the other animals. This observation suggests that some animals have the capacity to withstand high temperatures with less reduction in their thyroxine-secretion rate. If such animals do not show the usual seasonal decline in milk production and, further, can effectively dissipate the extra heat production which is associated with higher milk production, then selection of animals showing these characteristics might result in animals especially adapted to tropical climates.

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EFFECT OF MOLASSES AND BACITRACIN ON THE ESTROGENIC ACTIVITY OF SILAGE [1, 2]

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SUMMARY

The effects of zinc bacitracin alone and in combination with molasses on the preservation of grass-legume silage were investigated. Silage prepared with 4 to 40 g. of bacitracin per ton reached satisfactory pH levels, but pH remained above 5.0 when 400 g. per ton were used. In brome grass–alfalfa silage preserved with 40 or 80 lb. of molasses alone or in combination with 4 to 40 g. of zinc bacitracin per ton, there were no differences in protein, ether extract, fiber, or ash at the end of 90 days. The addition of molasses increased dry matter and nitrogen-free extract, and the combination of molasses and bacitracin tended to increase dry matter and NFE still more. Silage preserved with molasses had significantly more estrogenic activity than that with no preservative or with bacitracin alone.

Since the report of Lowe and Spohr *(2)* on the presence of estrogenic activity in plant materials, biologically active estrogens have been reported in at least 50 plant species. Pieterse and Andrews *(4)* found that alfalfa silage contained significant estrogenic activity and, that while there was considerable difference in activity between samples, alfalfa silage had significantly greater estrogen activity than freshly cut alfalfa. Stob *et al. (6)* attempted to account for the causes of increased estrogen activity during fermentation. However, when molasses or sodium bisulfite was used as preservative for alfalfa cut at different stages of maturity, there was no consistent difference in estrogenic activity between samples with or without preservatives. Since studies on the estrogenic activity of 56 strains of alfalfa *(5)* showed that considerable strain differences exist, it was suggested that the estrogenic activity of silage might be a function of the original activity of the forage *(6)*.

Because of the well-known effects of antibiotics on fermentation, it was decided to investigate the influence of antibiotics on fermentation of silage and its possible effects on estrogenic activity. A preliminary trial was carried out in the spring of 1957 with zinc bacitracin. During the summer of 1957, Dexter *(1)* reported on the use of several antibiotics in silage preservation. While there was a definite tendency for antibiotics to retard acid formation, silage made with bacitracin was of good color and odor *(1)*. It was decided, therefore, to carry out further studies with zinc bacitracin.

EXPERIMENTAL PROCEDURE

All silages were made by placing 2 lb. of freshly chopped forage in plastic bags, as described by Walker *et al. (7)*. The first experiment was carried out

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to determine if a satisfactory fermentation could be produced in the presence of zinc bacitracin. Second-cutting alfalfa in full bloom was harvested on June 13, 1957. One group of samples contained no preservative, one group contained ground corn at an equivalent rate of 200 lb. per ton of silage, and one group contained an equivalent of 80 lb. of molasses per ton. Zinc bacitracin was added at the rate of 4, 40, and 400 g. per ton of silage to samples with and without ground corn or molasses.

The second experiment was begun on August 14, 1957. The forage was a mixture of brome grass and alfalfa in the ¼ to ½ bloom stage. Molasses was added at levels of 40 and 80 lb. per ton of silage, and zinc bacitracin was added alone and in combination with molasses at levels of 4, 20, and 40 g. per ton.

The estrogenic activity was determined by the mouse uterine weight technique used in a number of experiments in this laboratory *(3, 4, 5, 6)*. The pH of the pressed juice was determined with a glass electrode pH meter and the chemical determinations by conventional analytical procedures for feedstuffs.

RESULTS AND DISCUSSION

As summarized in Table 1, it was established in a preliminary experiment that satisfactory fermentation did occur in alfalfa silage to which zinc bacitracin had been added. When no added carbohydrate was provided, the pH was still high at the end of 6 wk. The color of all samples was good. The odor of all samples with either corn or molasses was superior to those which had no added carbohydrate, but there was no gross evidence of putrefaction in any sample. When corn or molasses was added, highly satisfactory pH levels were reached within 2 to 4 wk. Zinc bacitracin did not interfere with acid production at a level of 40 g. per ton but, at the 400-g. level, it is apparent that the rate of fermentation was slowed. These results confirmed those of Dexter, although this was not known at the time.

TABLE 1

Effect of corn, molasses, and zinc bacitracin on the pH of alfalfa silage

Treatment	14 days	42 days
	(*pH*)	
Bacitracin		
0 g/ton	5.6	5.3
4 g/ton	5.6	5.3
40 g/ton	5.8	5.4
400 g/ton	5.9	5.7
Ground corn and bacitracin		
0 g/ton	4.8	4.4
4 g/ton	4.6	4.4
40 g/ton	4.7	4.3
400 g/ton	5.7	5.2
Molasses and bacitracin		
0 g/ton	4.1	4.1
4 g/ton	4.2	4.0
40 g/ton	4.6	4.4
400 g/ton	5.4	5.2

A second experiment was designed to study the possible relationship of mo-

lasses and zinc bacitracin, and to determine if bacitracin might replace or supplement molasses in silage preservation. The silage was a mixture of brome grass and alfalfa and was harvested on August 14, 1957. The pH, color, and odor were observed at intervals of 1, 3, and 6 mo. and the pH data are summarized (Table 2).

TABLE 2

Effect of zinc bacitracin and molasses on the pH of brome grass–alfalfa silage

Treatment	1 mo.	3 mo.	6 mo.
		(pH)	
Bacitracin			
0 g/ton	4.1	4.4	4.8
4 g/*ton*	4.0	4.1	4.9
20 g/ton	3.9	4.1	5.0
40 g/ton	4.6	4.0	4.8
Bacitracin plus 40 lb. molasses			
0 g/ton	4.5	4.2	4.6
4 g/ton	3.7	4.6	4.6
20 g/ton	4.3	4.4	4.2
40 g/ton	4.5	4.3	3.8
Bacitracin plus 80 lb. molasses			
0 g/ton	3.8	4.4	4.1
4 g/ton	4.0	4.3	4.0
20 g/ton	4.2	4.6	3.5
40 g/ton	3.6	4.2	3.8

It is apparent that a satisfactory pH was obtained in all samples, including those in which there was neither added molasses nor bacitracin. In Experiment 1 the degree of acidity obtained without added carbohydrate is more typical of what can be expected in the field, but it is recognized that fermentation sometimes proceeds satisfactorily in grass-legume silage without preservatives. The high level of zinc bacitracin (400 g/ton) was not used in the second experiment and, as before, 40 g. of bacitracin per ton did not interfere with acid production.

The chemical composition of the silage at the end of 90 days of fermentation is shown (Table 3). It appears that there are no important differences in protein, ether extract, fiber, or ash between any of the treatments. It would be expected that the use of molasses would increase both dry matter and nitrogen-free extract and this appears to be the case. There is a suggestion that the addition of zinc bacitracin to molasses may have further increased the dry matter and nitrogen-free extract. Since the determinations are on single samples of silage for each treatment, the significance of this observation must await verification by further testing. It is apparent, however, that zinc bacitracin does not prevent a highly satisfactory fermentation and preservation of nutrients in the presence of molasses, and that in Trial 2 silage was preserved satisfactorily without added carbohydrate.

After 3 mo. of storage, silage samples were dried, extracted in ethanol, and the extracts fed to ovariectomized mice for a three-day period. The increase in uterine weight is an index of estrogen activity, as previously reported *(3, 4)*. As summarized (Table 4), all silage samples showed estrogenic activity when they were compared to the standard control diet used in the mouse assay colony. When the data were grouped on the basis of molasses vs. no molasses, there was a highly

TABLE 3

Effect of zinc bacitracin and molasses on the composition of brome grass–alfalfa silage after 90 days of fermentation

Treatment	Dry matter	Protein	Ether extract	Fiber	N-free extract	Ash
	(%)					
Bacitracin						
0 g/ton	14.17	4.82	0.94	4.39	13.23	1.83
4 g/ton	13.15	4.71	1.22	4.48	11.93	1.57
20 g/ton	12.54	4.36	1.07	4.21	11.47	1.48
40 g/ton	13.28	4.87	0.87	4.43	12.41	1.78
Bacitracin plus 40 lb. molasses						
0 g/ton	14.60	4.50	0.77	4.55	13.83	1.86
4 g/ton	15.30	4.69	1.26	4.52	14.04	1.84
20 g/ton	15.22	4.57	0.94	4.13	14.28	1.80
40 g/ton	15.80	4.79	0.97	4.99	14.83	1.93
Bacitracin plus 80 lb. molasses						
0 g/ton	13.99	4.48	0.92	4.63	13.07	1.83
4 g/ton	15.35	4.52	1.07	4.42	14.28	2.01
20 g/ton	17.75	4.59	1.01	4.40	16.74	1.83
40 g/ton	17.37	4.72	1.42	5.10	15.95	2.03

significant increase in estrogen activity in the molasses groups ($P < 0.01$). The addition of zinc bacitracin at any level did not significantly affect estrogen activity in the absence of molasses. Although there was a significant interaction ($P < 0.05$) between molasses and antibiotic with respect to uterine weight, the

TABLE 4

Effect of zinc bacitracin and molasses on the estrogenic activity of brome grass–alfalfa silage after 90 days of fermentation

Treatment	Mice	Body wt.	Uterine wt.	Body wt.
	(No.)	(g.)	(mg.)	(%)
Bacitracin				
0 g/ton	5	26.00	37.20	0.142
4 g/ton	5	27.00	42.68	0.160
20 g/ton	5	27.20	37.40	0.138
40 g/ton	5	27.80	38.28	0.137
Bacitracin plus 40 lb. molasses				
0 g/ton	4	27.50	61.25	0.226
4 g/ton	5	25.80	48.04	0.188
20 g/ton	5	26.60	45.16	0.171
40 g/ton	5	27.20	48.32	0.179
Bacitracin plus 80 lb. molasses				
0 g/ton	5	25.10	35.80	0.144
4 g/ton	5	24.20	44.80	0.185
20 g/ton	5	27.00	44.52	0.169
40 g/ton	5	28.40	56.32	0.195
Control laboratory ration	5	25.60	25.32	0.099
Molasses effect				
0 lb/ton	20	27.00	38.89	0.144
40 lb/ton	19	26.74	50.14	0.189
80 lb/ton	20	26.20	45.36	0.173
Bacitracin effect				
0 g/ton	14	26.14	43.57	0.166
4 g/ton	15	25.67	45.17	0.178
20 g/ton	15	26.93	42.36	0.159
40 g/ton	15	27.80	47.64	0.171

variability within the molasses-antibiotic groups casts considerable doubt as to whether such a relation exists.

This paper contributes further evidence as to the complex and variable nature of the estrogen activity of feedstuffs. As summarized by Pieterse and Andrews *(3, 4)*, there are differences in estrogen activity associated with species, stage of

TABLE 5

Analysis of variance. Effect of zinc bacitracin and molasses on the estrogenic activity of brome grass–alfalfa silage after 90 days of fermentation

Source of variation	D.F.	Sum of squares	Mean square	F-ratio
Total	64	10,813.90		
Treatment	12	5,118.56	426.55	3.89[a]
Control vs. others	1	1,784.22	1,784.22	16.29[a]
Molasses	8	1,397.87	698.94	6.38[a]
Bacitracin	3	210.43	70.14	0.64
Molasses × bacitracin	6	1,726.03	287.67	2.63[b]
Error	52	5,695.34	109.53	

[a] $P < 0.01$.
[b] $P < 0.05$.

maturity, season of the year, and apparent differences in the fermentation of silage. Stob *et al. (5)* have found that individual alfalfa clones (strains) may vary greatly in estrogenicity, and this study contributes further evidence that silage may undergo changes in estrogen activity due to processing.

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EFFECT OF NATURAL COMBINATIONS OF AMBIENT TEMPERATURE AND RELATIVE HUMIDITY ON THE WATER INTAKE OF LACTATING AND NONLACTATING DAIRY COWS [1]

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SUMMARY

Four trials were conducted to measure the water consumption of dry and lactating cows under varying natural combinations of temperature and relative humidity. There was a highly significant correlation between water intake and temperature, but relative humidity did not have a significant effect upon water intake when temperature was held constant. A prediction equation was constructed using the regression of water intake on ambient temperature, and comparison was made between the predicted water intake of cows in this study and the water intake of certain cows determined from the tables prepared by other workers. Water consumption of cows kept under natural conditions appeared to have greater variation with the extremes of temperature than did that of cows kept in controlled chambers.

Studies of the ingestion by cattle of fluorides occurring naturally in water have confronted investigators *(2)* with the need for accurate estimates of the daily water consumption of cattle. The literature contains a number of reports giving estimates of water consumption of cattle and describing the effects of different factors that cause water consumption to vary. Rather thorough reviews of the subject have been published by Leitch and Thompson *(1)* and, more recently, by Sykes *(7)*. Based on data taken from the literature and from some unpublished data on feed and water consumption at Beltsville, Maryland, Winchester and Morris *(8)* worked out detailed tables of water consumption for cattle of different ages, taking into account the factors of ambient temperature, body size, level of feed intake and, in the case of lactating cows, butterfat test and daily milk yield. Most of the data available and used to develop the tables were recorded when cows were kept in chambers at nearly constant temperatures. Before depending entirely on the tables of Winchester and Morris for estimates of water consumption to be used in a study of intake of water-borne fluorides, it was decided to obtain data from an experiment where the animals were exposed to natural combinations of ambient temperature and relative humidity. Accordingly, an experiment was set up to study the effect of ambient temperature and relative humidity on the water intake of lactating and nonlactating cows kept in outside corrals.

METHODS AND PROCEDURE

Four separate trials with dairy cows were conducted. The trials began in April, 1957, and were concluded in July, 1957. Before each trial there was a

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three-day preliminary period, which was followed by the seven-day trial. Daily recordings were made of water intake, feed consumption, and milk production. Daily maximum and minimum temperature and relative humidity readings were obtained from the local office of the U. S. Weather Bureau (Lubbock, Texas).

In each trial, four dry cows (two Holsteins and two Jerseys) and four lactating cows (two Holsteins and two Jerseys) were used, making a total of eight cows per trial, and 32 different cows for the four trials. Mature cows were used except in Trial II, when it became necessary to substitute two heifers in advanced gestation because suitable dry cows were not available. The lactating cows generally were in medium-to-low production. The dry cows were all pregnant and in the latter part of gestation. An attempt was made in each trial to have four pairs, each pair consisting of a dry and a lactating cow, matching as nearly as possible in weight and breed.

The pens in which the cows were kept were in the open and without shade. In each pen was a 55-gal. barrel set approximately 12 in. in the ground to prevent its being upset. The barrels were individually calibrated by weighing exactly 400 lb. of water into each. The height of water was measured with a metal yardstick. The pounds of water per inch of depth then were calculated. Control barrels to measure evaporation were set and calibrated in like manner. Water consumed by each animal during each 24-hr. period (Table 1) was determined by measurement of the depletion in the barrel and by correcting for evaporation and rainfall. Measurements were made each day at the same time, except in Trial II on July 13 and 14, when readings were made too late in the day. In the statistical analysis the data of July 13, 14, and 15 were omitted.

The lactating cows in the first trial were fed sorghum silage, alfalfa hay, and a 16% protein concentrate mix. The dry cows were fed sorghum silage, alfalfa hay, and a 12% protein concentrate mix. In the second, third, and fourth trials sorghum fodder and green chopped alfalfa were substituted for the hay and silage.

RESULTS AND DISCUSSION

In Table 1, water intake is recorded as the average intake of total water by four cows, total water meaning the free water drunk from the barrels plus that ingested in the silage (66.89% moisture) or in chopped green alfalfa (77.86% moisture). To make the water-consumption figures more nearly comparable to those of the dry cows, 87% of the average daily milk yield of the lactating cows was deducted from the sum of the free water and the water in the feed. These figures for the lactating cows appear as "corrected daily total water intake." The water intake per 100 lb. of body weight was calculated by dividing the daily average intakes by the average live weight of the four dry or lactating cows. The average temperature and average humidity for each day were computed by averaging the two daily extremes of each.

Using the water intake per 100 lb. of body weight as the dependent variable, and temperature and relative humidity as the independent variables, the usual correlations and regressions were computed for the dry and lactating cows separately. These statistics are to be found in Table 2.

TABLE 1

The average daily water intake of nonlactating and lactating dairy cows

Trial	Date	Temperature	Humidity	Nonlactating cows		Lactating cows			
				Av. daily total water intake	Total water intake per 100 lb. body weight	Av. daily total water intake	Av. daily milk production	Corrected average daily total water intake	Total water intake per 100 lb. body weight
		(°F.)	(%)	(lb.)					
I	April 28	50	96	40	3	78	29	53	4
	29	55	90	49	4	84	28	60	5
	30	59	86	94	7	105	29	80	7
	May 1	63	88	77	6	127	27	104	8
	2	60	89	94	7	120	29	95	8
	3	62	84	62	5	117	28	93	8
	4	52	90	48	4	119	28	94	8
				Average weight, 1,338 lb.			Average weight, 1,235 lb.		
II	June 26	82	64	113	11	170	29	144	14
	27	80	53	130	13	185	30	159	16
	28	83	56	142	14	166	28	142	14
	29	89	50	143	14	196	26	173	17
	30	91	39	142	14	188	25	166	17
	July 1	88	52	145	14	220	24	200	20
	2	86	47	149	15	191	24	170	17
				Average weight, 1,019 lb.			Average weight, 999 lb.		
III	July 13[a]	82	43	119	10	140	20	123	12
	14[a]	82	41	182	16	215	22	196	19
	15[a]	80	45	121	10	152	21	133	13
	16	80	40	125	11	159	21	140	14
	17	81	39	138	12	162	20	144	14
	18	81	51	150	13	186	21	168	16
	19	81	57	124	11	156	18	140	14
				Average weight, 1,153 lb.			Average weight, 1,030 lb.		
IV	July 23	80	68	148	13	178	23	158	16
	24	76	76	99	9	150	25	128	13
	25	76	78	83	7	138	25	116	12
	26	80	65	120	11	155	25	133	13
	27	81	53	127	11	171	26	149	15
	28	81	50	146	13	191	24	170	17
	29	83	51	155	14	174	24	153	15
				Average weight, 1,140 lb.			Average weight, 1,005 lb.		

[a] Not used in statistical analysis.

A comparison of the correlations and regressions for the dry and lactating cows (Table 2) shows very close agreement in certain cases, and some divergence in others. The correlations between temperature and relative humidity ($r_{th} = -.90$) are the same for both groups because the data are the same. The correlations between temperature and water consumption ($r_{tw} = .94$ and .95) are similar for both groups, and the correlations between relative humidity and water consumption are also very close ($r_{hw} = -.90$ and $-.88$). A slightly greater divergence is noted for the correlations between temperature and water consumption, with humidity held constant ($r_{tw.h} = .68$ and .76), and a still greater difference for the correlations between humidity and water intake with temperature held constant ($r_{hw.t} = -.36$ and $-.18$); however, the latter values are not statistically significant. The multiple correlation coefficient, R, between water consumption and the two variables, temperature and humidity, is almost the same for the two groups of cows and is the same as the simple correlations between water consumption and temperature only.

The regression lines of Figure 1 indicate very similar responses of lactating

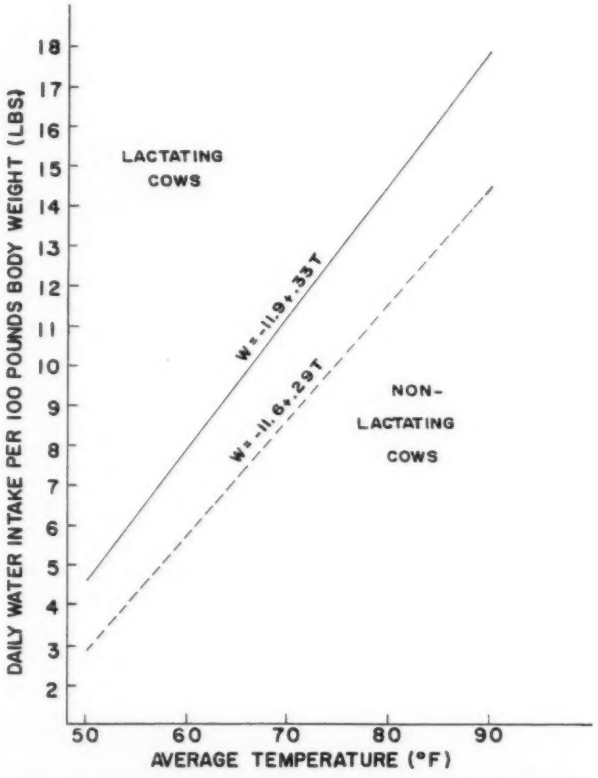

FIG. 1. Relationship between ambient temperature and water intake.

TABLE 2
Correlations and regressions

	Correlations			Regressions	
	Nonlactating cows	Lactating cows		Nonlactating cows	Lactating cows
r_{th}	−.90[a]	−.90[a]	b_{wt}	.29[a] ± .02	.33[a] ± .02
r_{tw}	.94[a]	.95[a]	b_{wh}	−.18[a] ± .02	−.20[a] ± .02
r_{hw}	−.90[a]	−.88[a]	$b'_{wt.h}$	.68[a] ± .17	.83[a] ± .15
$r_{tw.h}$	.68[a]	.76[a]	$b'_{wh.t}$	−.28 ± .17	−.13 ± .15
$r_{hw.t}$	−.36	−.18	$b_{wt.h}$	.21[a] ± .05	.29[a] ± .05
R	.94[a]	.95[a]	$b_{wh.t}$	−.06 ± .03	−.03 ± .03

[a] Significant at the 1% level.
t, temperature; h, humidity; w, water consumption per 100 lb. body weight, av. of four cows.

and dry cows to temperature changes, but they also indicate a difference in the water-consumption rates for the two groups. This difference may be explained, at least in part, by differences in rates of dry matter intake in the two groups. The lactating cows had an estimated daily dry matter intake of 2.59 lb. per 100 lb. of live weight and the dry cows averaged 2.01 lb. Winchester and Morris *(8)* take dry matter intake into account in their water-consumption table, and they also distinguish between the water-consumption rates of lactating and nonlactating cows. It appears that lactating cows, being on a higher nutritional level, would drink more water per 100 lb. of live weight, exclusive of that secreted in milk, but not necessarily more water than nonlactating cows per unit of dry matter intake.

The correlations between water consumption and environmental temperature ($r = .94$ and .95 for dry and lactating cows) are rather surprisingly high, in view of the findings of others. Woodward and McNulty *(9)* found that cows fed similar rations and kept inside a barn drank about the same amount of water in moderate weather as in cold weather, but when the weather became hot, water consumption increased 79%. Three pairs of Holstein cows kept by Reagan and Mead *(6)* inside a psychrometric room at temperatures from 40 to 100° F. and at constant relative humidity (60%) showed uniform water consumption from 40 to 80° F., but above 80° the cows actually reduced their water consumption. Under similar controlled conditions, and at temperatures rising from 50 to 105° F., Ragsdale *et al. (3, 4)* also found that water consumption remained fairly constant up to 80° F. From 80 to 105° F., certain cows, but not all, would decrease their water consumption. While much of the previous work would indicate some type of association between temperature and water consumption, none of it would suggest that the association is a simple linear one. Conditions under which data were obtained varied from the laboratory chambers of Reagan and Mead *(6)* and Ragsdale *et al. (3, 4)* to the barn-fed and maintained animals of Woodward and McNulty *(9)*, then to the animals kept in outside corrals, in the present study.

The negative correlations between water intake and relative humidity (−.90 and −.88, Table 2), for the dry and lactating cows, respectively, are highly significant ($P < .01$), but when temperature is held constant the correlations drop to −.36 and −.18, neither of which is statistically significant. The highly sig-

nificant correlations of −.90 and −.88 between water intake and relative humidity probably came about mostly because of the strong association between humidity and temperature and because the latter was strongly correlated with water consumption. Apparently, relative humidity did not have a powerful causal relationship with respect to water consumption. The standard partial regression coefficients ($b'_{wt.h}$ and $b'_{wh.t}$, Table 2) suggest that temperature is much the more important factor in water consumption, yet the possibility that relative humidity under some conditions may affect water consumption is not completely ruled out. In studies under controlled conditions, Ragsdale *et al. (5)* showed that cows consumed more water under low humidities and less water under higher humidities, but at temperatures below 75° the effect of relative humidity was not significant. At temperatures above 75° the effect of humidity increased. If this effect of relative humidity holds true for natural combinations of temperature and humidity, as reported in Table 1, then it is probable that relative humidity had little influence on water consumption in the first trial, where all daily average temperatures were in the 50's and 60's, but it may have helped to increase water consumption slightly in Trials II, III, and IV, when daily mean temperatures usually ranged from 80 to 90° and humidity was at the lowest. No drop in water consumption at temperatures above 80° F. was noted as reported by Reagan and Mead *(6)* and Ragsdale *et al. (3, 4, 5)*, who worked under controlled conditions.

For predicting water consumption per 100 lb. of live weight, a simple equation making use of the regression of water intake on temperature alone would be about as accurate as the multiple regression equation taking into account both temperature and relative humidity. Simple regression equations for the regression lines of Figure 1 are as follows:

$$\text{Lactating cows:} \quad W = -11.9 + .33\,T$$
$$\text{Nonlactating cows:} \quad W = -11.6 + .29\,T$$

W is the estimated water consumption per 100 lb. live weight and T is the average of the daily maximum and minimum temperatures. Such equations, of course, are useful only within the range of temperatures of this study. Water consumption rates probably decline only very slowly with declining temperatures below 50° F., and they may also decline instead of increase at temperatures above 90° F. In such cases, the above prediction equations could lead to large errors in predicting water consumption. On the other hand, the average of the daily maximum and minimum temperatures rarely exceeds 90° F. in this locality.

Winchester and Morris *(8)* made a comparison of the actual consumption of water in gallons of certain cows as reported by Ragsdale *et al. (3, 4)* and the values predicted for the same cows from the Winchester and Morris tables on water consumption. The prediction was based on ambient temperature, body weight, daily milk yield, and butterfat test. The values taken from the Winchester and Morris tables were in close agreement with the actual water consumption figures reported by Ragsdale *et al.* For example, one cow weighing 1,190 lb. and producing 34.3 lb. of 4.1% milk drank 21 gal. of water at the temperature of 80° F. The tabular estimate for the cow was also 21 gal. If predicted from the regression line for lactating cows (Figure 1) in this study, the amount for such a cow would be estimated at 172.5 lb. of water, to which would be added 29.8 lb.,

the amount secreted in the 34.3 lb. of milk. The total then is 202.3 lb., or 25.8 gal. For another cow, with the temperature at 70° F., Winchester and Morris estimate 21 gal., the equation of Figure 1 estimates 20 gal., and the actual consumption reported by Ragsdale *et al.* was 22 gal. At temperatures in the 50–60° range, the equation of Figure 1 yields a considerably lower estimate for water intake than does the Winchester and Morris table. The regression line of Figure 1 yields higher water consumption estimates for the higher temperatures, but near the same estimates for temperatures near 70° F.

It is not clear why cows under natural conditions should be more extreme in their responses to natural variations, unless certain temperatures and relative humidities or other unknown factors combine to operate in the same direction at the extremes. The lower temperatures noted in this study were accompanied by the higher humidities. In fact, in the first trial, April 28 to May 4, there was considerable rainfall which wet the cows. Any air movement during the time the cows were wet may have operated to produce in the animal a reaction more extreme than that in animals in chambers at the same temperature-humidity combination. A combination involving wind movement may account for the divergence of water consumption from that reported where cows were kept in chambers under controlled temperature and relative humidity. At the other extreme, low relative humidity which has been shown to be effective at temperatures above 75° *(5)*, may have combined with temperature to boost water consumption above that observed under controlled conditions. The effects of such combinations at the extremes of temperature would tend to maintain the linearity of the temperature-water relationship throughout the range of these data.

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REPEATABILITY ESTIMATES OF PER CENT TOTAL PROTEIN AND CERTAIN OTHER CONSTITUENTS OF THE MILK OF HOLSTEIN COWS AT SELECTED INTERVALS OF LACTATION[1]

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SUMMARY

The objectives were to obtain estimates of individual cow repeatability of the major milk component groups and to obtain information relative to the importance of the frequency of sampling milk. These estimates were based on the performance of 12 Holstein cows that completed a 36-day test regime and five other Holstein cows that completed the first 24 days of the test. The relative frequency of sampling necessary to estimate the production of each milk constituent studied was indicated by the importance of the three components of variance. An analysis of variance procedure was used to determine estimates of repeatability of pooled individual cows within months (R_1) and over months (R_2). The milk component groups and their respective R_1 and R_2 values were: *(a)* % milk fat (0.84 and 0.99); *(b)* % solids-not-fat (0.99 and 0.92); *(c)* % total solids by Mojonnier (0.99 and 0.88); *(d)* % total solids by Cenco (0.89 and 0.80); *(e)* % total protein (0.75 and 0.60), and *(f)* daily milk yield (0.33 and 0.17). Linear correlation coefficients were obtained on individual cows and then pooled over cows between the data concerning milk production and between these factors and certain weather data. These coefficients were low, indicating the possibility that these factors might be varied independently in a breeding program.

During the past decade, there has been much discussion of the milk-pricing system and means of altering it for the mutual benefit of the producer, processor, and consumer. Likewise, interest has increased in the possibility of changing the relative amounts of solids in milk.

If such marketing changes were made, it would be desirable to adjust the breeding program to produce a product having maximum value. Specifically, if some constituent other than milk fat were to become a criterion of value, then the breeder would need to be able to apply successfully selection pressure for that constituent. Since this must be possible if progress is to be made by the breeder, it is essential that these constituents be evaluated with respect to their repeatability.

The data that have been reported in the literature *(1, 3, 5, 6, 9, 11, 13,* and others) show relatively high coefficients of correlation between the major milk constituents. These values have been obtained by evaluating milk samples obtained by one or more of the following sampling patterns: *(a)* one or more days composite sample of herd milk taken once each month over an extended period of time; *(b)* a one or more days' composite sample of the milk of in-

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dividual cows taken once a month over an extended period of time; *(c)* two or more days' composite sample of the milk of individual cows taken every 5 wk. over an extended period of time; or *(d)* a one-day composite sample of the milk of cows once a week or some other stated time interval over an extended period of time. In such sampling procedures, the daily variation of the individual cow would be minimized. Consequently, it was deemed desirable to examine the daily variation in the major constituents of milk periodically throughout the lactation of individual cows, in order that the relative importance of this source of variance might be assessed.

EXPERIMENTAL PROCEDURE

Seventeen winter-calving Holstein cows that had calved prior to March 1, 1954, and were in the same general stage of lactation, were selected for this testing program. A milk-sampling schedule was arranged so that each cow would be intensively sampled during March, June, and September. In each sample month, each cow was to have a one-day composite of night and morning milk taken for four consecutive days in each of three periods. Period 1 consisted of the third, fourth, fifth, and sixth; Period 2 consisted of the 15th, 16th, 17th, and 18th, and Period 3 consisted of the 24th, 25th, 26th, and 27th days of the month. The four-day periods within each month were selected by chance and the months were chosen so that coverage of the entire lactation would be as broad as possible under such a sampling program. Therefore, each sample month could be used both as a general stage of lactation and as a season of the year.

Twelve of the cows completed the 36 scheduled sample days, while the other five completed only the first 24 days. A total of 552 individual cow, daily composite samples were taken. These cows were maintained in a dry lot operation with limited access to pasture. The percentages of the following components were determined: *(a)* total protein (TP) *(2)*; *(b)* milk fat (F) *(12)*; *(c)* total solids by the Mojonnier method (TS_m) *(12)* and by the Cenco method (TS_c) *(10)*, and *(d)* solids-not-fat (SNF) by difference from the Mojonnier procedures. Daily milk yield (DMY) in pounds per sample day; averaged barometric pressure; maximum and minimum air temperature, and solar radiation in Langley units were concomitantly recorded. The atmospheric data were taken over the 24-hr. period during which the milk was produced.

Standard statistical procedures were followed throughout *(14)*. An analysis of variance procedure was used to obtain the components of the total variance due to: *(a)* consecutive days within a four-day period; *(b)* periods within the month, and *(c)* months for each cow and factor related to the milk that was produced. The data were analyzed as obtained and were not corrected for age or computed to cover the total milk produced during the entire lactation. The equation, $R_1 = \frac{\sigma_e^2}{\sigma_e^2 + \sigma_p^2}$, was based on variances within cows and within months. R_1, σ_e^2, and σ_p^2 represent repeatability estimate, variance due to days, and variance due to periods, respectively. The equation, $R_2 = \frac{\sigma_e^2 + \sigma_p^2}{\sigma_e^2 + \sigma_p^2 + \sigma_m^2}$

was based on variances within cows and over months. R_2 and σ_m^2 represent repeatability estimate and variance due to months, respectively. All repeatability values were obtained for individual cows, then pooled over cows, to obtain a single within-cow estimate for each factor by each method cited above.

Estimates of correlation coefficients were established between the milk constituents examined and between those constituents and total milk yield and the weather data previously mentioned. Coefficients were calculated for individual cows, then pooled over periods within months and over months, to obtain an estimate of the coefficient of correlation that would be most applicable as a lactation estimate under the conditions of this sampling schedule. The Chi-square test was used to evaluate the differences between correlation coefficients of a given set of data *(14)*.

RESULTS AND DISCUSSION

Pooled estimates of repeatability for individual cows for each of the methods of calculation are shown (Table 1).

TABLE 1

Pooled within-cow repeatability estimates and relative importance and distribution of daily, periodic, and monthly components of variance

Factor (%)	Repeatability		Number of times each source of variance was of greatest importance for R_2								
			Day			Period			Month		
	R_1	R_2	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
F	.84	.99	15	2	0	0	9	8	2	6	9
SNF	.96	.92	16	1	0	0	5	12	1	11	5
TS_m	.99	.88	13	4	0	0	3	14	4	10	3
TS_c	.89	.80	10	6	1	0	5	12	7	6	4
TP	.75	.60	4	5	8	0	11	6	13	1	3
DMY	.33	.17	0	6	11	0	11	6	17	0	0

It was evident from the estimated mean square of the analyses of variance, for both per cent of fat and SNF, that day and period were more important as sources of variation than were months. Total solids showed the same trend as fat and SNF, by both the Mojonnier (TS_m) and Cenco procedures (TS_c). It was of interest to note, however, that daily variation appeared to be greater by the Mojonnier procedure than by the Cenco procedure.

Differences between months were the greatest sources of variation for most of the cows with respect to the per cent of total protein and daily milk yield. Periodic and daily variation were of lesser importance. This might have been anticipated with respect to daily milk yield. The distribution of the importance of the sources of variance for each factor measured is shown in Table 1. The size of R_1 parallels this distribution. Therefore, it would appear to be necessary to sample all of the constituents of milk that were examined here more frequently than daily milk yield, to obtain production estimates for a given period of time that were equally accurate for all factors. The relative frequency of sampling required for each factor will vary as the importance of the daily, periodic, and monthly component of variance changes.

The pooled estimates of the linear correlation coefficients between the milk constituents studied and between each of these constituents and total milk yield and atmospheric data are shown (Table 2). Those coefficients which exceeded the Chi-square value of 0.05 were included to give an indication of the extent to which those traits were related.

TABLE 2

Pooled linear coefficients of correlation

Factors	r	Factors	r	Factors	r	Factors	r
1, 2	−.135	2, 4	.326[a]	3, 7	.001	5, 6	.089[a]
1, 3	.453[a]	2, 5	.129	3, 8	−.063	5, 7	−.183[a]
1, 4	.304	2, 6	.111	3, 9	.036	5, 8	−.184[a]
1, 5	.049	2, 7	−.134	3, 0	.135	5, 9	−.211[a]
1, 6	−.022[a]	2, 8	.025	4, 5	.206	5, 0	−.026[a]
1, 7	.197	2, 9	.098	4, 6	−.060[a]	6, 7	−.130[a]
1, 8	−.161	2, 0	.091	4, 7	−.002	6, 8	−.122[a]
1, 9	−.125	3, 4	.607[a]	4, 8	−.114	6, 9	−.119[a]
1, 0	.114	3, 5	.127	4, 9	−.075	6, 0	.031
2, 3	.814[a]	3, 6	.062[a]	4, 0	−.094		

1 — % F
2 — % SNF
3 — % TS_m
4 — % TS_c
5 — % TP
6 — pound DMY
7 — Average barometric pressure
8 — Maximum temperature (° F.)
9 — Minimum temperature (° F.)
0 — Solar radiation (gm. cal/min/cm^2)

[a] Exceeded Chi-square value at 0.05.

The correlation between F and SNF attained its highest absolute value during the month of June, or just past mid-lactation for most of these cows. The negative relationship between these factors is contrary to the findings of Cranfield *et al. (4)*, but agrees with the data of Houston and Hale *(8)* that these two factors do not vary together. The correlation between fat and total protein fluctuated very closely about zero. This value is much lower than those reported by others *(1, 5, 7, 13)*. Reasons for this difference may be in the frequency with which the animals were sampled, the fact that one-day samples were used rather than composites of two or more days at less frequent intervals, and a possible real difference in the populations sampled due to breed, selection program, and environmental conditions which may have exerted natural selection pressure on the cattle involved. These pooled coefficients represent individual cow relationships rather than some other and perhaps broader basis.

These correlations indicate the possibility of altering the composition of the SNF fraction of milk without affecting the SNF–TS relationship.

The relationships between weather data and factors concerning milk production were found to be small and somewhat variable as judged by the number of estimates of correlation that exceeded the Chi-square value of 0.05.

The correlations between F and barometric pressure were all positive in character, whereas those between SNF and barometric pressure were all negative. Total solids, by both procedures, were essentially independent of barometric pressure as judged by the pooled estimates of correlation. Total protein and DMY were both negatively correlated with barometric pressure, but the number of coefficients exceeding Chi-square at 0.05 reduces their usefulness.

The general relationship between maximum and minimum temperature and factors related to milk production was negative. The only pooled estimate that was positive in both instances was SNF and this did not differ markedly from zero in either instance. With the exception of SNF, the results obtained by correlating solar radiation to the factors concerned with milk production were similar to those obtained by relating barometric pressure to the same set of factors. In the correlation of SNF and solar radiation the coefficient was of the same general magnitude as for SNF and barometric pressure, but different in sign. The reason for such a change in sign was not to be found in the available data.

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TECHNICAL NOTES

RESULTS OF A BLOOD TEST FOR BRUCELLOSIS AND LEPTOSPIROSIS IN A LARGE DAIRY HERD

A history of low breeding and excessive abortions of calves during the spring and summer months and during the 7th mo. of pregnancy, in a large Texas dairy herd, led the authors to suspect that cattle in the herd were infected either with brucellosis or with leptospirosis or both. The disease management program in this herd had been lax. For example, natural service to bulls untested for reproductive diseases had been followed until recently. Also, calfhood vaccination for brucellosis control had not been practiced until the last 2 yr. It was felt that the breeding efficiency of cows in the herd might be improved, and the calf crop increased, by culling animals found to be positive to brucellosis and/or leptospirosis blood tests. There was a possibility, in addition, of answering the question put forth by researchers and livestock raisers, "Is there a tendency for dairy cows to have a general susceptibility to more than one infectious disease?" The evidence provided by this test to answer the question is presented below.

Blood samples were taken from 399 breeding-age females in the herd on February 27, 1958. The samples were tested in the Texas Agricultural Experiment Station Veterinary Research Laboratories for the presence of *Brucella abortus* by the procedure of Eiselle *et al. (1)*, and for *Leptospira pomona* by the plate agglutination method of Stoenner *(2)*.

Of the 399 females blood-tested, 83 reacted positively to the brucellosis test and 71 reacted positively to the leptospirosis test. Of this group, 15 animals reacted positively to both tests. Of the total number tested, only 129 cows were negative to leptospirosis, with 199 considered as suspicious reactors. The 83 positive reactors to the brucellosis test represented 20.8% of the number tested, while 17.8% of the 399 cows tested reacted positively to the leptospirosis test.

Fifteen individuals, or 3.76% of the 399 cows tested, reacted positively to both diseases. The product of the percentages of positive reactors to each separate disease is 3.7%. This percentage of reactors to both diseases would be expected by chance alone. It will be noted that the actual percentage of reactors to both diseases is in close agreement with the expected. From the limited number of animals included in this test, there is no evidence to indicate that dairy cattle having a specific susceptibility to an individual infectious disease are likely to show a general susceptibility.

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A COMPARISON OF EGG YOLK–SODIUM CITRATE, EGG YOLK–SODIUM CITRATE–GLYCINE, AND EGG YOLK–GLYCINE AS SEMEN DILUTERS

Recently, Roy and Bishop[1] reported that a mixture of equal parts of egg yolk and a 4% solution of glycine preserved semen for a longer period of time than did equal parts of egg yolk and a 2.9% citrate buffer. However, in microscopic preparations the sperm are more easily seen in egg yolk–citrate than in egg yolk–glycine. Storage studies with ram semen at this station have shown that a mixture of egg yolk–citrate and glycine improves the storage life of the sperm. On the basis of this finding, studies were made of the comparative value of egg yolk–sodium citrate, egg yolk–sodium citrate–glycine, and egg yolk–glycine as diluters for the semen of buffalo bulls, bovine bulls, and rams.

[1] Roy, A., and Bishop, M. W. H. Effect of Glycine on the Survival of Bull Spermatozoa in Vitro. *Nature*, **174**: 746. 1954.

MATERIALS AND METHODS

There were ten samples of semen from four bovine bulls (Hariana), ten samples from three water-buffalo bulls (Murrah), and seven samples from four rams (Bikaneri), during December, January, February, and March, 1955-56. The motility rating, hydrogen ion concentration, and sperm density were determined on each sample within 30 min. after collection by routine methods in this laboratory. Only those samples rating +3 or higher were used in the storage studies.

Each semen sample was divided into three portions and each portion preserved in one of the following diluters, prepared as follows:

Egg yolk–sodium citrate–glycine. An aqueous solution of 1.8% sodium citrate dihydrate and 2% glycine was mixed with egg yolk in equal parts by volume.

Egg yolk–glycine. A 4% aqueous solution of glycine was mixed with egg yolk in equal parts by volume.

Egg yolk–sodium citrate. A 3.6% aqueous solution of sodium citrate was mixed with egg yolk in equal parts by volume.

The bovine semen was diluted at a rate of 1:11, the buffalo semen at a rate of 1:5, and the ram semen at a rate of 1:25. These rates of dilution were determined in part by the sperm concentration of the samples.

The diluted semen samples were stored at 4° C. and examined for rate of motility (+5 representing the highest rate of motility and +0 representing no motility) every third day until the motility of the samples had decreased to a little above +3. From then until the rating was a little below +3, it was examined every day. After the +3 grade was reached, the motility was rated on alternate days until all the sperm were dead.

RESULTS AND DISCUSSION

Table 1 and Figure 1 show the rather striking superiority of the glycine-containing mixtures as compared to the conventional egg yolk–sodium citrate mixture. The analysis of variance of the data for both the bovine and the buffalo bull semen indicated a statistically significant difference between the glycine-containing mixtures and the nonglycine-containing controls, but no significant difference between the two glycine-containing mixtures. For the ram semen, the mixture of egg yolk–sodium citrate–glycine was significantly superior to the mixture of egg yolk–glycine, which was significantly superior to the egg yolk–sodium citrate control.

It is of interest to note also that the mixture of egg yolk–sodium citrate and glycine allowed the sperm in one sample of ram semen to remain motile for 54 days, the longest period that has yet been reported in the literature for ram semen, so far as these authors have been able to determine.

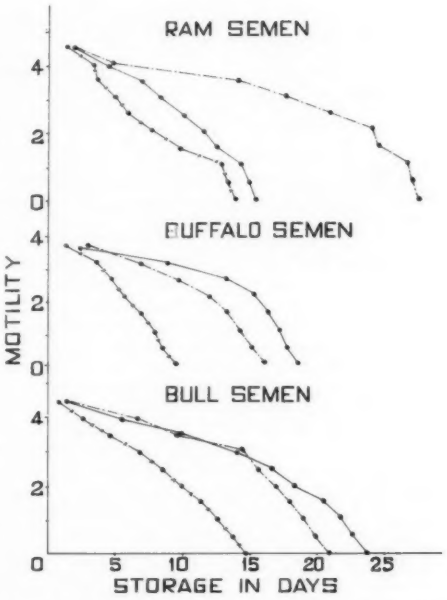

FIG. 1. Decline in motility of spermatozoa for semen stored at 4° C. in different diluters.

Decline of motility of semen stored at 4° C. in egg yolk–sodium citrate—[•–×–•–×–•].

Mixture of egg yolk–sodium citrate and glycine—[•– – –•– – –•].

Egg yolk–glycine—[•———•———•].

In further studies on the duration of livability in storage under summer conditions (the preceding data being obtained under winter conditions), the maximum length of survival of ram sperm has been 17 days in the egg yolk–sodium citrate–glycine diluter.

SUMMARY

The average length of time that a +3 motility was maintained in egg yolk–citrate–glycine, egg yolk–glycine, and egg yolk–citrate, was 14.7, 13.9, and 6.8 days, respectively, for bovine sperm; 6.8, 8.8, and 3.5 days, respectively, for buffalo sperm; and 17.2, 8.0, and 4.4 days for ram sperm. The egg yolk–sodium citrate–glycine mixture gave better visibility of the sperm in microscopic preparations.

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TABLE 1

Maintenance of the motility of spermatozoa in the semen of three species in different diluters

Sample No.	Initial motility	Sperms in millions per ml.	pH	Days that sperms maintained motility between initial and +3			Days for initial motility of sperms to reach +0		
				Egg yolk–glycine	Egg yolk–sodium citrate and glycine	Egg yolk–sodium citrate	Egg yolk–glycine	Egg yolk–sodium citrate and glycine	Egg yolk–sodium citrate
Bovine semen									
1	+4	1.45	6.5	18	14	9	22	19	20
2	+4	1.50	6.7	20	17	8	53	23	12
3	+4	1.40	6.7	14	14	3	21	19	8
4	+4	1.88	6.4	14	20	5	18	24	11
5	+4.5	2.05	6.4	15	13	7	20	20	13
6	+4.5	1.20	6.8	10	20	8	25	26	14
7	+4	0.98	6.4	11	15	7	19	22	18
8	+4.5	1.76	7.0	14	14	9	19	20	18
9	+4.5	2.20	6.4	14	11	5	23	13	14
10	+4.5	1.34	6.4	9	9	7	16	21	16
Average	+4.25	1.57		13.9	14.7	6.8	23.6	20.7	14.4
Buffalo semen									
1	+3.5	1.60	7.0	5	5	9	19	19	17
2	+4	1.53	7.0	14	14	6	19	18	16
3	+3.5	1.88	7.0	15	16	5	20	21	6
4	+3	1.18	6.8	12	7	1	19	15	6
5	+3	1.78	6.7	1	1	2	9	8	6
6	+3	1.10	6.8	8	8	3	18	15	8
7	+3.5	0.67	6.8	3	3	1	21	19	9
8	+3.5	1.04	7.0	15	5	6	19	13	15
9	+3	1.40	6.4	5	6	1	18	14	6
10	+3	0.54	6.7	10	3	1	23	18	7
Average	+3.30	1.27		8.8	6.8	3.5	18.5	16.0	9.6
Ram semen									
1	+5	3.45	6.5	12	40	4	18	54	7
2	+4.5	3.60	6.4	3	11	3	5	18	11
3	+4.0	3.62	6.5	9	28	6	13	33	22
4	+5.0	3.82	6.5	12	15	6	18	27	15
5	+5.0	5.06	6.4	5	12	3	11	20	13
6	+4.0	3.42	6.4	8	8	5	13	26	14
7	+3.5	3.55	6.4	7	7	4	28	12	12
Average	+4.42	3.78		8.0	17.2	4.4	15.1	27.1	13.4

OUR INDUSTRY TODAY

GROWTH AND DEVELOPMENT OF THE DAIRY INDUSTRY IN THE UNION OF SOUTH AFRICA

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Although the Union of South Africa does not play a prominent part in the international dairy trade, the local industry—despite certain limitations of climate—has shown a marked increase in the volume of production during the past half-century. This growth forms part of the general evolution of what was purely a pastoral land, barely 90 yr. ago, into the most highly industrialized portion of Africa.

Local dairy commodities at present include butter, various types of cheese, and condensed and powdered products, in addition to pasteurised, sterilised, and cultured milks and ice cream. A brief outline of dairy production since the Union was established in 1910 is given in Tables 1 to 4, which record values for certain representative years, such as the beginning and end of World War II. Some details of imports from South West Africa (S.W.A.) and the British Protectorates of Bechuanaland, north of the Union, and Swaziland to the east, are included (Tables 2 and 3). (The third Protectorate, Basutoland, enclosed by Union territory, has no dairy plants.) A system of reciprocal free trade exists between the Union and these adjoining territories *(16)*. All the butter and Cheddar cheese that they manufacture in excess of their domestic sales is disposed of by the South African Dairy Industry Control Board, in the same way as Union production. Table 1 shows that, in terms of whole-milk equivalents, the most important outlet for primary producers since Union has been the butter industry, followed by the city fluid-milk market, with cheese and condensed and powdered milk always far behind. The statistics (Table 5) in regard to Cheddar and Gouda varieties cover at least 98% of the Union's cheese output, the remainder being mainly Blue-Mould, i.e., Roquefort type. Very limited quantities of soft cheese and other varieties are made. The proportion of Gouda in the combined Union manufactures (Table 3) increased from between 25.6 and 28.7% in 1940–44, to 37.0% in 1955–56.

TABLE 1

Percentage utilisation of South African milk (except whole milk used in rural areas)

	Butter[a]	Cheese[a]	Condensed and powdered milk[b]	Fluid milk (urban)[c]
1910–11	53.5	1.3	....	45.4
1919–20	56.4	6.4	....	37.2
1929–30	56.0	7.2	2.7	34.1
1939–40	59.7	7.5	4.5	28.3
1945–46	43.6	7.4	4.0	45.0
1949–50	47.7	7.1	5.3	39.9
1955–56	50.4	7.7	6.4	35.5

[a] Estimated from weight of manufactured products. Butter: correction made for surplus butterfat received from other sections of industry.

[b] From Economics and Markets and Dairy Board data.

[c] From Economics and Markets data. Ref. *(2)* and unpublished.

Tables 2, 3, and 4 show various aspects of the marked rise in dairy output over the 46 yr. covered. Thus, combined factory and farm production of butter (Table 2) increased by 682%, and of cheese (Table 3) by 4,806%. The average rates of increase per annum of butter and cheese production in the first four decades of Union were 5.6 and 5.4%, respectively *(16)*. An important advance has been the long-term decline in farm manufacture. Although the first creamery was opened in 1890 *(1)*, production on the farms still exceeded that in the factories in the early period of Union—butter in 1910–11 only, and cheese in four of the years between 1910 and 1916 *(16)*. While farm production showed some further increase, e.g., in butter until about 1940, its contribution to the total output declined from 54.3 to about 8.5% of butter, and from 58.7 to about 0.4% of cheese, during the period covered by the tables. Only 30% of the farm butter made during 1949–50 was actually sold *(23)*, which proves that these once-prominent products are vanishing from the market. Until the early part of World War I, approximately 25% of the butter and 85% of the cheese requirements were imported *(16)*. Since 1930 in particular, local products have been exported from time to time.

The Union was a consistent net importer of condensed milk until 1938, and of milk powder even shortly after World War II *(32)*. While attempts to establish a local industry prior to 1910 were unsuccessful, manufacture developed to a limited extent between 1918 and the late 1920's *(24)*. The increase in condensery and

TABLE 2

Long-term trends in the butter industry of South Africa

	Production		Imports from			
	Factory[a]	Farm[b]	S. W. Africa and Protectorates[a]	Other countries	Exports[a]	Consumption[a] Union (factory butter)
	(1,000 lb.)					
1910–11	5,191	6,159	.....	+3,262[c]		8,453
1919–20	10,864	6,945	.....	− 278		10,586
1929–30	18,180	11,266	.....	−3,167		15,013
1939–40	44,810	(12,150)	11,635		18,117	38,150
1945–46	33,810	9,907	5,914		230	40,138
1949–50	55,469	8,120	11,002		624	64,909
1955–56	81,237	(7,500)	8,181		3,152	83,198

[a] Dairy Board data. Exports: 1939–40 onwards include re-exports ex S. W. Africa, etc. Exports in 1950–51, 1953–54, and 1954–55 exceeded 4 million lb.

[b] 1939–40, Ref. *(32)*; 1955–56 estimate; remainder—Agr. Census.

[c] Net imports (+) or exports (−)—Ref. *(16)*; separate details for butter (and cheese) ex S. W. Africa, etc., prior to 1931, not available.

TABLE 3

Long-term trends in the cheese industry of South Africa
(Cheddar and Gouda varieties)

	Production		Imports[a] from			Consumption[a] Union (factory cheese)
	Factory[a]	Farm[b]	S. W. Africa and Protectorates	Other countries	Exports[a]	
	(1,000 lb.)					
1910–11	225	320	.....	+4,639[c]		4,864
1919–20	3,782	272	.....	− 135		3,647
1929–30	7,217	446	.....	−1,289		5,928
1939–40	13,980	278	337	166	3,104	11,251
1945–46	14,541	330	.....	.....	79	14,542
1949–50	18,447	353	185	.....	46	18,066
1955–56	26,633	(108)	229	.....	162	26,145

[a] See Table 2. Exports during 1953–54 and 1954–55 exceeded 2.3 million lb.

[b] Refs. *(16, 22, 32)*; 1955–56: estimate.

[c] See Table 2.

TABLE 4

Some long-term aspects of South African milk production

	Urban	Rural	Condensery and drying plant intake[a]	All milk: Total[b]	All milk: Output per capita *(lb.)*	Total inhabitants[c] 1,000's
	Fluid milk[a]					
	(1,000,000 lb.)					
1910–11	224	443	.....	934	158	5,925
1919–20	271	513	.....	1,241	183	6,782
1929–30	413	594	31	1,806	220	8,215
1939–40	618	563	99	2,751	268	10,264
1945–46	1,030	622	91	2,909	256	11,361
1949–50	1,194	632	158	3,624	294	12,329
1955–56	1,390	639	249	4,557	330	13,792

[a] See Table 1. Rural: Farms and Bantu areas.

[b] Including butter and cheese, but not calf-feeding.

[c] From Census Office mid-year estimates, 1955–56: Whites, 2,881; African (Bantu), 9,233; Asian, 416; Coloured, 1,262.

powder plant milk intake between 1929–30 and 1955–56 (fourth column, Table 4) was 702%. During 1955–56, when manufactures of condensed milk amounted to 61.2 million lb. and, of milk powder, to 8.9 million lb., the respective increases on the corresponding 1942–43 values were 102 and 205%. In contrast to the United States *(30)*, a high percentage of the condensed milk output of South Africa consists of the sweetened variety (99.7% in 1949–50 and 93.7% in 1955–56). Its flavour and keeping qualities make it most popular with the lower-income groups, especially among the non-Whites.

Between 1910–11 and 1955–56, the total milk output, i.e., excluding amounts fed to calves, increased by 388% (Column 5, Table 4). If rural consumption (Column 3) is excluded, this is equivalent to 697%. However, the corresponding increase in the total human population, given in the last column, is 133%. Thus, dairy production has undergone the more rapid advance. The resultant increase in annual output per capita (shown in Column 6) is also reflected in both butter and cheese production, the corresponding values for 1910–11 and 1955–56 being 1.9 and 6.4 lb. and 0.1 and 1.9 lb., respectively.

General economic development. Until approximately 1880, the South African economy was predominantly pastoral, with agriculture largely at the subsistence stage *(31)*. The discovery of diamonds in 1867 and, more particularly, of gold in 1886, created big markets for farm products. Agriculture was rapidly commercialised, but mining became a far more predominant economic force. Following World War I, a third important factor appeared, viz., secondary industry. After the more or less world-wide depression in the 1930's, gold- and base-metal mining expanded again, but industry—and, at this stage, heavy industry—made big advances, stimulated by the demands of World War II. After this war, new gold- and uranium-mining centres arose, but the rapidly growing manufacturing industries developed export markets, especially in the adjoining territories, and successfully challenged mining as the leading sector of the national economy. The rapid rise in the total population (noted in Table 4) obviously is due to these over-all trends, which caused the proportion of residents in urban areas to increase from 25.5 to 42.6% between 1911 and 1951, and provided the domestic stimulus to dairy expansion.

Agro-climatology (33). The greater part of South Africa consists of a vast inner tableland, almost entirely enclosed within the 18° C. isotherm, and fringed by a narrow coastal plain. Mean annual temperatures are as low as 10–12° C. along the eastern escarpment, but they exceed 20° C. in some of the low-lying portions of the inland and coastal areas. In the interior plateau, great temperature fluctuations, both daily and seasonal, are common. Figure 1 shows that, in general, the mean annual rainfall (national average 475 mm.) diminishes inland Vfrom the south and southeast coasts and from east to west across the interior. The total area actually can be divided as follows, according to the mean annual rainfall and its effectivity:

(a) 30.3% is arid—less than 250 mm. (mainly shrub and desert grass).

(b) 34.4% is semiarid—between 250 and 500 mm. (mixed scrub grass in western elevated plains and savanna in lower subtropical regions further north).

(c) 35.3% is more humid or temperate—above 500 mm. (includes elevated eastern portions of the inland plateau, mostly in the 1,000-mm. belt and dominated by *Themeda-Andropogon* grassland, as well as by coastal and inland forests).

About 86% of the country, mainly the eastern and northern parts, has a predominantly summer-rainfall season. The more arid western areas are prone to droughts, which are common, too, in the more favourable easterly regions. The southwest Cape Province (Area A, Figure 1), comprising 11% of the Union, enjoys winter rains; whereas, in the remaining 3% (the restricted south-coastal belt where Port

FIG. 1. Dairy areas of South Africa in relation to mean annual rainfall zones. Main fluid-milk markets: CT (Cape Town); PE (Port Elizabeth); EL (East London); DBN (Durban); JHB (Johannesburg and remainder of Witwatersrand); PTA (Pretoria); BFN (Bloemfontein).

Main manufacturing milk and cream areas	(% Union production 1955–56) Butter	Cheese	Condensery and drying plant milk intake
A. S. W. Cape	9	17	12.3
B. E. Cape	11	4	1.5
C. N. E. Cape and S. Free State	9	13	.....
D. E. Free State–E. Transvaal	23	26	57.4
E. Natal–E. Griqualand	8	8	24.0
F. Central and N. Transvaal	16	3	.....
G. N. W. Cape	24	29	4.8

Elizabeth is situated) the rainfall is more or less evenly distributed throughout the year.

From FAO statistics, the percentages of the Union's surface[1] used for farming, etc. were estimated as follows: (Corresponding U.S.A. values are given in parentheses) *(11)*

	(%)	
Arable land	7.5	(24.1)
Permanent meadows and pastures	75.7	(32.7)
Irrigated land	0.4	(1.5)
Forest land	0.9	(33.1)

Regional development. The relationship between dairying and other sections of agriculture, and the competition between the various divisions of the dairy industry for the farmers' supplies (Table 1), will explain the present regional setup of this traditionally intensive enterprise (Figure 1). Climatic factors obviously have made the major part of the Union pre-eminently suited to extensive pastoral production (wool, slaughter stock, etc.), which accounted for approximately one-third of the gross value of the national farm output *(18, 19)* between 1949 and 1956. The mean gross value during 1953–56 was equal to $950.8 million (U.S.A.) and the leading contributors were: maize, 16.5%; wool, 16.0%; primary dairy products, 11.3%, and slaughter cattle, 8.6%. Maize was first in 15 of the 20 yr., 1936–56, and wool in the remainder. (Primary dairy production was third in 13 of these years; during 1942–49 it was second.) The better rainfall regions have had to cater to all types of primary production, some perhaps more remunerative than dairying. Thus, it is not surprising that they have not satisfied the total demand for milk.

The following relationships between the mean prices per 100 lb. milk or whole-milk equivalent, paid by the various sections of the industry, are based on Economics and Markets *(2)* and Dairy Board data:

	1937–40	*1955–56*
Fluid milk equals	100	100
Condensed and powdered milk	75.1	62.8
Cheese	55.6	59.1
Butter[a]	51.7	47.8

[a] Intake—cream.

When there is a shortage, manufacturers obviously have to yield supplies to the fluid-milk markets, which in turn off-load any surpluses on the former. Condenseries similarly draw on the intake of the cheese factories. The fluid-milk supplier, often situated on relatively expensive land, must satisfy city health department requirements and must be prepared to meet constant consumer demands, even in the dry months when feed costs are high. The differentiation in prices also recognises a trend, observed in many countries, for butter and cheese—the former in particular—to pave the way for condensed and dried milk, and perhaps, finally, for city milk supply. This complete trend has been of importance mainly in the higher-rainfall areas in which the largest cities are all situated (Figure 1). Area E (Natal–E. Griqualand), for example, had the lead in regional cheese output until 1940–45, and in condensery and drying plant intake until recently, but its dairy production is now dominated by the ever-increasing demands of Durban, the major commercial port of South Africa.

Despite the fact that city milk-sheds, e.g., of Johannesburg and Pretoria, encroach into Area D (E. Free State–E. Transvaal), the details in regard to Figure 1 show that it leads the country in dairy manufacture, especially of condensed and powdered milk. Its butter and cheese totals increased by 99 and 76%, respectively, between 1940–45 and 1950–55. During the latter period, it was the foremost cheese manufacturing region and a close second to the N.W. Cape (Area G) as regards butter. Area D is a leading crops region (maize, etc.) and shows the most promise in regard to future dairy expansion.

The most important factor in South African dairying is, however, the big regional development of the semiarid N.W. Cape (Area G). During each five-year period since 1940–41, it has averaged 23 to 26% of the Union's butter output; whereas, its share of the cheese output increased from 11 to 24% between 1940–45 and 1950–55. It can boast three out of the five largest creameries, as well as the largest cheese factory, and has a history of butter and (farm) cheese manufacture dating back to the early 1900's. Here many farmers dispatch cream (often testing 50% butterfat or more) only once a week or, in the low-production months, even less frequently. Some cream suppliers make a crude casein from skimmilk. In the cold, dry months cheese milk is collected on some routes only every second day. Over a long period, a heavy percentage of the local farmers has been unable to obtain sufficient land to make a satisfactory living from beef cattle exclusively. They have either to depend on dairying or to sell out.

The so-called "dairy ranching" system is almost entirely dependent upon the natural vegetation—the sparse grass cover and certain edible shrubs and bushes—as well as upon the deep underground water supplies. The notoriously low phosphorus content of soils and vegetation necessitates the use of bone meal and similar supplements. The erratic rainfall, already rather poor, makes it difficult to organise calving to promote maximum production

[1] About 472,359 sq. mi., or 15.6% of the area of the U.S.A.

and, together with the vulnerability of the shallow soil to wind erosion, makes successful feed crop cultivation impossible. The poor carrying capacity—much more than 15 acres per animal—can scarcely be improved.

Semiarid dairying has made Union butter and cheese production more vulnerable to droughts, but its contribution to national food supplies can not be abandoned lightly. The fact that since 1910 about half the primary output has gone to the butter industry (Table 1) is also in keeping with the extensive nature of much of the Union's dairy farming.

Seasonal variations. Throughout the country there is a marked seasonal variation in the amounts of nutrients available to stock from the natural vegetation *(32)* and this is reflected in dairy production trends. Rörich *(16)* shows that during the period 1940–52 the extreme monthly variations in national butter output, expressed as percentages of the mean for the 12 single months, were approximately 140% in January-February-March and 63% in September. The corresponding values for cheese were 129% in January and 78% in June and in September. Even greater fluctuations for these products were noted during the 12 preceding years, 1928–1939.

To encourage production during the dry, or poor rainfall, months, the Dairy Industry Control Board introduced the present extensive schemes of paying winter premiums on cream for butter-making and cheese milk (in addition to the particular basic price) in 1940. No doubt this has contributed towards the narrowing of seasonal trends, but it is questionable whether any further reduction can be expected in the semiarid areas.

On the fluid-milk market the degree of such variation is far less *(7)*. This part of the industry is not controlled by the Dairy Board, and to ensure an adequate supply, city dairies or marketing organisations have introduced a quota system. In general, a producer's quota for which he is guaranteed the official price decided for the city, depends on his output during the months of lowest production.

Procedures and milk output. White farmers owned approximately 2.9 million cows between 1953 and 1954, compared with 1.9 million in 1911. In 1949–50 *(25)* about 64.5% of such cows were kept mainly for milking purposes. Friesians accounted for 58.2% of the dairy cattle in 1950–51. Following in popularity were the Jerseys, 14%, Dairy Shorthorns, 8.2%, and Red Polls, 7% *(26)*. The long-term increase in milk production noted in Table 4 does not appear to be due to any significant rise in the mean yield per cow *(6)*, which is rather low *(20, 25)*. There has been a tendency also for the bulked supplies of the condenseries *(4, 6)* and the Cape Town fluid-milk plants *(5)* to decline both in fat and in S.N.F. content.

The Dairy Industry Control Board regulations make it possible for both cheese factories and condenseries to pay for milk according to the pounds of butterfat it contains. During 1950–53, only about 20% of the Union's cheese milk was bought on a butterfat basis, averaging, however, 3.745% fat *(16)*, compared with 3.47% for the country's fluid milk supplies all purchased on a quantity basis *(6)*. Therefore, the Board is considering a new compulsory scheme for manufacturing milk, under which the price per 100 lb. will vary according to the fat content.

African (Bantu or Negro) farmers supply manufacturing plants accessible from their own areas, which cover 12.9% of the country's surface *(21)* and are mainly used for agricultural purposes. Although no data are available on their contribution to the total output, it does not appear to be high. Only 754 of the Union's 67,759 farm separators in use during 1949–50 were in their possession. In 1954, they owned 41% of the country's 11.6 million cattle, both inside and outside their own areas, but the number of cows actually milked was probably very limited. The greater majority are small producers. At one factory, they constituted 53% of the suppliers in 1957 but accounted for only 11% of the intake, mainly in quantities of less than 10 lb. daily. The members of cooperative milk production schemes sponsored by the Union Native Affairs Department (Agricultural Section) own an average of two cows each. It is interesting to note that butter-making was practised by some of the tribes before contact was made with the early White settlers *(1)*. The main contribution of the African to the dairy industry is not as a primary producer but as a general labourer in the factory and on the farm.

The State and the industry. The first railways catered to the mines, but after Union, in 1910, when the new central government took over the complete network, many branch lines were constructed in the farming areas *(9)*. The subsidiary road motor services, which were greatly extended in the 1920's, fostered cream production in the most remote areas, especially in the N.W. Cape. Milk and cream, in common with other agricultural commodities, are conveyed by the Administration at reduced rates. Apart from cream, however, dairy plants undertake the collection of a heavy percentage—in many cases all—of their supplies. Tanker collection from country depots is extensively developed in the Durban milk-shed.

In the Department of Agriculture, widespread extension, research, and educational services deal with problems on the farm, and specialised services cater to the dairy industry. South Africa has been plagued by many cattle diseases *(13)*. Without the Onderstepoort Veterinary Laboratories, the animal industry would be in a backward, if not hopeless, state. Official

measures to combat the severe erosion of farming land were crystallised in the Soil Conservation Act of 1946. Such work is assisted by the Agro-Economic Map, which divides the country into more or less homogeneous agricultural regions in harmony with environmental conditions *(27)*.

Since 1934, only bulls which have been approved by departmental inspectors may be kept in proclaimed cattle improvement areas, which today include most of the farms. Official milk-recording or cow-testing commenced in 1917. It was considerably extended in 1953–54 under a new scheme providing for cooperative organisations *(3)*. Although the effect on commercial herds in the past has been limited—even in 1955 only about 1.5% of the Union's dairy cows were tested *(3)*—the development of artificial insemination services may spread its influence.

Farmers' cooperatives for the processing and distribution of dairy products have been fostered by legislative action. During 1953–55, these bodies produced about 48% of the butter, and 63% of the cheese, output. They also have a big share of the fluid-milk market, but do not own any condenseries. The Dairy Industry Act No. 16 of 1918, now replaced by Act No. 63 of 1957, was the first major dairy law. This legislation provides for the registration and supervision of manufacturing plants by the Department of Agriculture. The production and distribution of fluid milk is mainly administered by the city health departments.

Controlled marketing. The severe economic depression which followed World War I caused butter and cheese factories drastically to cut prices for butterfat and milk. Producers retaliated by manufacturing butter themselves. The Board of Trade and Industries found that the manufacture and marketing of dairy products during these years were hampered by high factory costs, by the unjustifiably large number of plants, and by the mistrust of farmers *(24)*. Thus, a Dairy Industry Control Board, dealing specifically with butter and cheese, was created in 1930, and concerned itself mainly with the subdised export of these products.

The far-reaching Marketing Act, which provides for the introduction of marketing schemes for various agricultural products to be administered by boards with regulatory and price-fixing powers, was passed in 1937. In 1939, the Dairy Board was reconstituted under this Act, with powers to control fluid milk, but certain distributors successfully challenged the scheme in the Supreme Court and it was declared *ultra* vires. The whole fluid-milk industry remained outside the control imposed by the Marketing Act until 1956, when distribution Cape Town fell under the newly constituted Cape Milk Board. Local producers may sell only milk or fresh cream to distributors registered with this Board, which imposes quotas and fixes prices *(23)*. Price fixation in the other large centres is the responsibility of the Government Price Controller.

The national Dairy Industry Control Board has operated since 1940 under a Marketing Act scheme which was amended again in 1954, and which now applies to primary producers and manufacturers in the butter, cheese, and condensed and powdered milk industries. No concern may manufacture any of these commodities without a Board licence. Manufacturers, consumers, and the Department of Agriculture are represented on the Board, but in common with all such bodies constituted under the Act, the producers are in the majority. There are at present 17 commodity control boards, and their activities are coordinated by the National Marketing Council. To finance its activities and to build up reserves, the Dairy Board imposes levies on butter and cheese factories according to their output, and on condenseries and powder plants according to their intake. It determines the prices for milk and butterfat used in those sections of the industry under its control, as well as the wholesale and maximum retail prices for butter and Cheddar, Gouda, and process cheese. It also has to decide on manufacturing margins for butter and cheese, i.e., the difference between the cost of the raw material and the final return. The imposition of levies and any price-fixation measures are subject to the approval of the Minister of Agriculture, to whom appeals may be directed if any manufacturing licence is refused. Condensed and powdered milk determinations fall under the Government Price Controller, but neither these products nor cheese types other than Cheddar and Gouda are subject to marketing control.

Under the Dairy Board, producers' prices have increased markedly and more rapidly than the retail prices for butter and cheese. In apparent response, the volume of production of manufacturing milk and cream has increased more rapidly than that of fluid milk and, especially, more rapidly than that of other pastoral products *(15)*. The proportionate share of manufacturing and marketing to the cost structure of butter has also been reduced considerably; whereas, the share of primary production has increased greatly. The producer received about 63% of the retail price of butter between 1927–30 or 1934–38, in contrast to about 81% during 1950–53. For cheese, the corresponding figures were about 45 and 70% *(16)*. Butter and cheese processing costs declined between 1935–38 and 1950–51 by approximately 30 and 40%, respectively.

Improved transport facilities and the Board's registration policy have discouraged the indiscriminate erection or operation of plants. The resultant larger output per unit has undoubtedly been the biggest factor in reducing costs. The 72 creameries operating during

1927–30 averaged 221,000 lb. butter per annum, compared with 45 averaging 1,632,000 lb. during 1953–54. In the cheese industry, the corresponding values were 145 and 45,000 lb. and 59 and 472,000 lb. *(16)*. During 1955–56, 14 of the 56 cheese factories manufactured more than 500,000 lb.; five of the 45 creameries more than 4 million lb. butter, and seven of the 26 condenseries or drying plants had a milk intake of more than 18 million lb.

Some plants manufacture more than one product. Buttermilk and separated whey, which until recent times created serious disposal problems in country areas, are now powdered by some concerns, whereas others divert these materials to their own piggeries. During 1955–56, about 1.9% of the Union's butter was made from whey cream.

In the fluid-milk market, similar developments have occurred. Since the late 1930's, both the producer's price and his share of the higher retail price of milk have been greatly increased, and there has been a big swing from the many small raw milk dairies towards fewer and larger processing plants. In Cape Town, there is 100% pasteurisation of supplies today. In six other large centres, between 75 and 98% is heat-treated (pasteurised or sterilised). Thus, apart from the often criticised powers of the Dairy Board to refuse manufacturing licences, it must be realised that the tendency towards fewer units of greater output and efficiency is a normal economic development. To some extent, price increases for primary products have resulted from a general upward trend. The Board, however, has effected a greater stabilisation of prices.

In addition to the need for cutting costs, technical advances can be ascribed largely to general world improvements in processing equipment and the assistance offered by the Department of Agriculture and the State-sponsored national research organisation, the Council for Scientific and Industrial Research, which also controls the South African Bureau of Standards. The industry employs few of the already small number of graduates in dairy science from the three local faculties of agriculture. Process control and factory laboratory organisation are fields in which big progress still can be made.

Disposal of products. Although operating pools for all butter and Cheddar and Gouda cheese produced in the Union or received from S.W. Africa and the Protectorates, the Dairy Board delegates the actual marketing to its agents, and factories receive their shares of the proceeds of the particular pool according to their pro rata production. The grading of the total manufactures by government dairy officers has been enforced since 1940. Under its exclusive rights, the Board imports butter only when local stocks can not satisfy demands, such as happened between 1941 and 1945. Cheese imports, also subject to a duty, usually are limited to types not manufactured locally.

Between 1910–11 and 1955–56 the per capita consumption of butter increased from 2.5 to 6.6 lb., and of cheese from 0.9 to 1.9 lb., per annum. Data up to 1950–51 show that the annual rate of increase in the consumption of butter exceeded the rate of production; for cheese, the reverse appears to be true *(16)*. During World War II, the big demand and the effect of droughts resulted in rationing of supplies to distributors. However, at ruling prices the public has been unable consistently to absorb the total stocks. Apart from regular supplies to ships and usually to adjacent territories, the first heavy shipments of butter and cheese overseas since the 1930's were dispatched by the Board between 1950 and 1955. (See Tables 2 and 3.) South Africa has built up a permanent export trade in primary and secondary agricultural commodities such as wool, fruit, and fruit products. Dairy exports, however, depend wholly on any surplus at home.

Such sporadic supplies can never compete satisfactorily with those of specialised exporting countries. In addition, the history of the Union's overseas trade in butter and cheese since the 1930's has been a history of losses which have tended to diminish, however, in recent years *(16)*. These losses have been met occasionally by reducing the manufacturers' margin or the producers' price, but mainly from the Board's levy funds.

The need to increase home consumption is fully realised. The Government butter subsidy instituted in 1943 has represented about 9.0% of the retail price since 1953–54, but earlier, when the price was slightly lower, it represented more than 11%. Winter premiums on butterfat are paid to producers from this subsidy, the proportion used for this purpose increasing fro mabout 50 to nearly 67% between 1951–52 and 1954–55. During the months when premiums are paid, the total return to the producer for the butterfat contained in a pound of choice butter exceeds the wholesale price for the latter.

Large-scale assisted consumption schemes have been developed. The former State-aided Milk and Butter Scheme, administered by the Dairy Board between 1935 and 1944, provided milk and cheese free to certain children, and butter, at lower prices, to some lower-income groups. Such butter is now handled by the Department of Nutrition and present recipients pay 13.1% less than the already subsidised market price. Since 1940, however, the percentages of butter and cheese stocks sold through such schemes have dropped heavily. Milk and other dairy products are among the foodstuffs distributed free to White, Coloured, and Asian children participating in present official schemes. The cost of enriching brown bread with milk solids and other nutrients is borne by the State. The present utilisation of

solids from skimmilk, buttermilk, and whey powders and casein for this purpose amounts to about 4.3 million lb. per annum.

Ordinary tertiary manufacture is also an important user of milk solids. The annual output of process cheese has shown no definite upward trend, fluctuating between 1.2 and 1.6 million lb. during the past 13 yr. The ice cream total, however, increased from nearly 1.1 to 4.2 million imperial gallons per annum between 1950–51 and 1953–54, accounting for 2.0–2.5% of the butter consumption. Between 1945–46 and 1953–54, the combined utilisation of fluid milk and concentrated milk products by the local baking, confectionery, and ice cream industries was equivalent to between 3.5 and 4.7% of the solids present in the original whole milk intake of all city dairies, condenseries, and drying plants.

The local dairy industry, in common with those of many other countries, has strongly opposed the manufacture of a margarine resembling butter, especially as regards colour, and this has influenced Union legislation since the first Dairy Act of 1918. Except for a brief period between 1945 and 1950, only white margarine has been permitted. During recent years, a national quota of 18 million lb. per annum has been imposed *(8)*, but total output up to 1956 had never reached 13 million lb. in any single year. The present retail price is 40% lower than that of choice (first-grade) butter. Margarine is an important item in the State-aided food schemes and, during periods of shortages, has replaced butter entirely. During 1954–55, about 29% of the output was distributed through these channels. Such stocks were sold at 33⅓% lower than the ordinary retail price in 1957. According to the food balance sheets of the Union *(14,28)*, butter formed 43.6% of the available fats and oils in 1953; 47.5% in 1954, and 50% in 1955. Its use is largely confined to the table and to ice cream manufacture.

In 1949, du Plessis *et al.* showed that in relation to the nutritional requirements of the total population, the shortfall in milk supplies for all purposes except butter-making was 47.8%; whereas, the shortfall for total fats was 122.5% *(10)*. Recent FAO statistics *(11)* show that, in regard to estimated calorie and animal protein contents of national average food supplies per capita, South Africa occupies a position lower than that of North America and certain European countries, but higher than that of some South American countries and those of the Far East.

All the assisted consumption schemes have improved nutritional standards and, incidentally, introduced new consumers to dairy products, but only a limited section of those who could benefit by the greater use of such products has been reached. African (Bantu) purchases of dairy produce are largely confined to whole, skim, or buttermilk, or sweetened condensed milk. Very few use butter or cheese. Their staple diet is often maize—a poor source of protein. The need to substitute a high-quality animal protein, e.g., from dried skimmilk, has been stressed by nutritionists *(17)*.

While the population is steadily increasing, past developments have shown that, in the Union's expanding economy, production must tend to increase at a far greater rate, i.e., more milk should be available per capita. There is no doubt that production in the more humid areas could be increased greatly, especially during the months when pastures are in best condition. The State irrigation schemes, even in the more arid areas, offer big potentialities. Van de Wall and Alvord, in summing up the food and feed resources of South Africa until 1946, estimated rather conservatively that it would be possible to increase the livestock population by approximately 30–50% by adopting better methods of pasture management, etc. *(32)*. Henning *(12)* has also stressed the need for increasing the average yield per cow and the fertility rate, as well as for obviating the big annual losses of cattle through malnutrition, and of calves both through disease and through slaughterings. He has shown how urgently needed improvements in veterinary services can greatly increase production. Informed opinion thus is aware of present shortcomings and of the great future possibilities. However, increased production must be linked with the most efficient and economical utilisation of the country's milk solids output. This can be achieved only by improving the regulation of the milk flow and by the coordination of the different sections of the industry, to provide *inter alia* for the purchase of all milk on a quality basis.

SUMMARY

Since the Union of South Africa was founded in 1910, the volume of dairy production and consumption has increased—both in total amount and per capita. This growth is part of the evolution of the purely pastoral land of the last century into a predominantly mining, and, finally, into a predominantly industrialised, economy. Although dairy development is hampered by the fact that over 30% of the country is arid and another 34% semiarid, the latter portion now contributes at least 24% of the butter and 29% of the cheese output. Production of thees two commodities, as well as of condensed, dried, and fluid milk, also has increased greatly in the more humid areas on which sound future development depends. Official assistance is responsible for much of the progress made by dairying in general, but world advances in equipment design also have been important.

State-sponsored control of marketing, which dominates the agricultural economy, has resulted in the one-channel schemes of the Dairy

Industry Control Board in regard to national butter and cheese output, and of the new Cape Milk Board in regard to fluid milk in one centre. Dairy Board policy has resulted in the more rapid advance of producers' prices for manufacturing milk and cream as compared with retail prices for final products. The producers' share of the increased retail prices for butter and cheese has also risen, but proportionate manufacturing costs have been reduced. Commercial butter and cheese manufacture on the farm—a predominant factor over 40 yr. ago—is slowly disappearing. Although affected by the Dairy Board's licensing policy and improved transport facilities, the tendency for fewer processing units of greater capacity—also observed in the fluid-milk trade—is a normal economic development. When South Africa has been unable to absorb its total butter and cheese stocks, including receipts from S.W. Africa and the Protectorates, at ruling prices, the Dairy Board has exported the surplus, usually at a loss. Various assisted consumption schemes have used some of these excess stocks, and it is obvious that in the still-expanding industrial economy the future of dairying must depend heavily on the large potential home demand which can be met through the better utilisation of natural resources and the resulting improved milk solids output.

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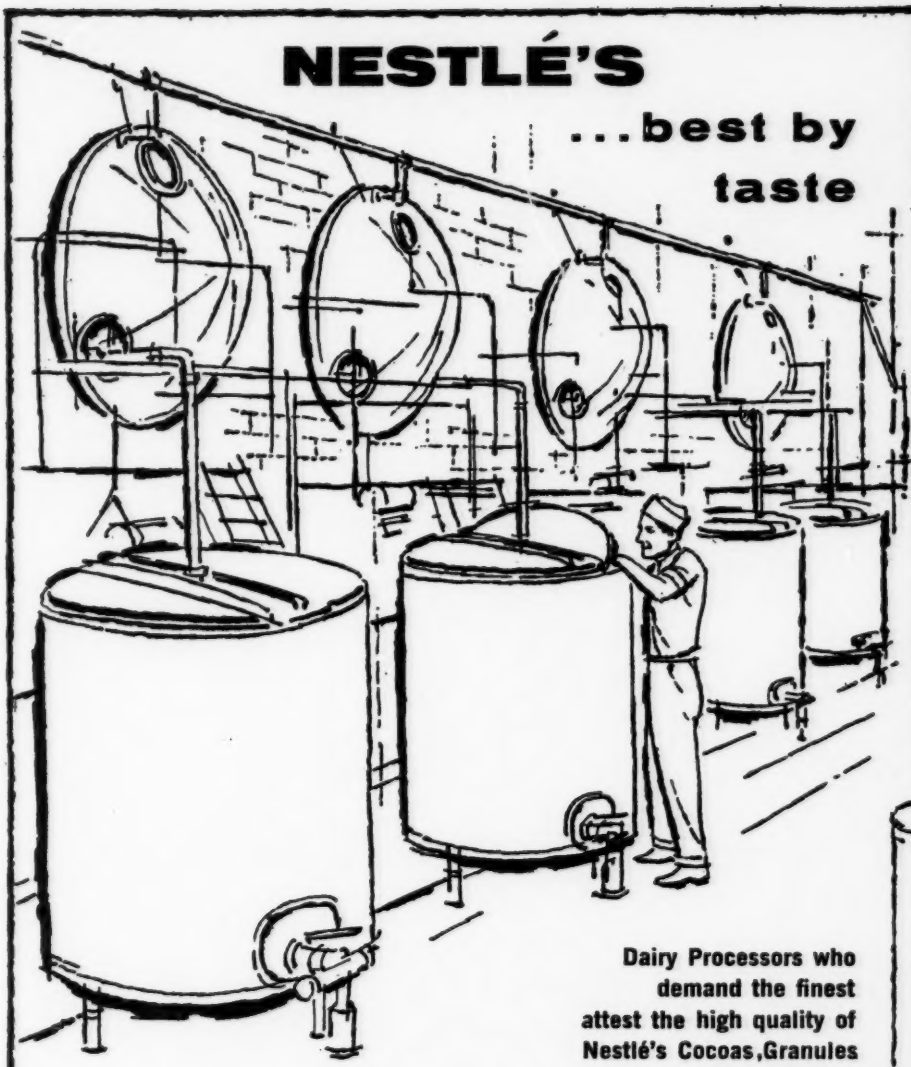

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